A STUDY OF BIOFILM FORMATION AND VANCOMYCIN RESISTANCE AMONG ENTEROCOCCAL SPECIES IN A TERTIARY CARE HOSPITAL

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BONAFIDE CERTIFICATE

This is to certify that the dissertation entitled **"A STUDY OF BIOFILM FORMATION AND VANCOMYCIN RESISTANCE AMONG ENTEROCOCCAL SPECIES IN A TERTIARY CARE HOSPITAL"** submitted by **Dr.R.BEAULA LILLY** to the Tamil Nadu Dr. M.G.R. Medical University, Chennai in partial fulfillment of the requirement for the award of M.D degree Branch– IV (Microbiology) is a bonafide research work carried out by her under direct supervision & guidance.

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This is to certify that the dissertation **"A STUDY OF THE BIOFILM FORMATION AND VANCOMYCIN RESISTANCE AMONG ENTEROCOCCAL SPECIES IN A TERTIARY CARE HOSPITAL."** is a bonafide record of work done by **Dr.R.BEAULA LILLY,** under my guidance and supervision in the Institute of Microbiology, Madurai Medical College, Madurai during the period of her Post graduate study of M.D. MICROBIOLOGY from 2012 – 2015.

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DECLARATION

I, **Dr.R.BEAULA LILLY** declare that, I carried out this work on, **"A STUDY OF BIOFILM FORMATION AND VANCOMYCIN RESISTANCE AMONG ENTEROCOCCAL SPECIES IN A TERTIARY CARE HOSPITAL"** at the Institute of Microbiology, Madurai Medical College. I also declare that this bonafide work or a part of this work was not submitted by me or any others for any award, degree or diploma to any other University, Board, either in India or abroad.

This is submitted to The Tamilnadu Dr. M. G. R. Medical University, Chennai in partial fulfillment of the rules and regulations for the M.D. Degree examination in Microbiology.

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CONTENTS

A STUDY OF BIOFILM FORMATION AND VANCOMYCIN RESISTANCE AMONG ENTEROCOCCAL SPECIES IN A TERTIARY CARE HOSPITAL

ABSTRACT

Aims and objectives:

Enterococci have emerged as an important nosocomial pathogen and possessed many virulence factors.This study was carried out to detect the biofilm forming ability and the prevalence of Vancomycin resistance among clinical Enterococcus species isolated in various samples collected at Govt.Rajaji Hospital,Madurai Medical College.

Materials and Methods:

The biofilm production in Enterococcal species was evaluated by three phenotypic methods such as Tissue culture plate(TCP) method,Tube method and Congo red agar(CRA) method. Vancomycin Resistant Enterococci(VRE) isolates presumptively identified by vancomycin screen agar were subjected to Microbroth dilution method for the determination of Vancomycin and Teicoplanin MIC and Polymerase

Chain Reaction(PCR) for phenotypic and genotypic detection of Vancomycin resistance type respectively.

Results:

Among 142 Enterococcal isolates,Tissue culture plate method detected 52 (36.62%) biofilm producer than Tube method 40 (28.17%) and Congo red agar method 31 (21.83%) . Tissue culture plate method was found to be more sensitive (100%) and specific(97.82%) than Tube method and Congo red agar method for the detection of biofilm formation in Enterococcal isolates. Among 19 VRE isolates presumptively identified by Vancomycin screen agar ,16 isolates were identified as Van B type and remaining 3 isolates were identified as Van A type both by phenotypic MIC detection method and genotypic PCR method.

Conclusion:

Tissue culture plate method was proved to be a simple and cost effective method for the early diagnosis of biofilm formation in the Enterococcus species.In molecular method limited settings,phenotypic classification of Vancomycin resistance by the determination of Vancomycin and Teicoplanin MIC by Microbroth dilution method can

be recommended. Appropriate surveillance, stringent infection control measures and continuous monitoring is very important to control the horizontal transmission of Vancomycin Resistant Enterococci.

Keywords:Biofilm,Tissue culture plate,VRE,Broth microdilution.

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INTRODUCTION

Until last decade, Enterococci were considered as organisms of low virulence. Recently, Enterococci are the fast emerging organisms causing serious nosocomial outbreaks. This is due to acquiring of virulence factors and multidrug resistance of the pathogen. This organisms ability to easily accumulate, acquire and share extrachromosomal elements encoding virulence traits or antibiotic resistance genes leads to their increased environmental survival and their nosocomial pathogenicity.

Enterococci are normal residents of oral cavity, gastrointestinal and biliary tracts and in lower numbers seen in vagina and male urethra. They are considered as opportunistic pathogen.[40] Enterococci are the common pathogen that cause the nosocomial urinary tract, intra abdominal and pelvic regions, blood stream, surgical sites, neonatal and central nervous system infections.[71]

The term ente'rocoque was used in an article in the French literature in 1899.[45]Thiercelin coined the term "enterococcus" to state bacteria in pairs and short chains seen in human stools. The

organisms was initially designated 'Micrococcus zymogenes' because of its fermentative properties.[45] Andrews and Harder in 1906,used the name Streptococcus faecalis to describe an organism of fecal origin which fermented lactose and mannitol but not raffinose. Streptococcus faecium, a second organism which differed from the fermentation property of S.faecalis was described by Orla Jensen in 1919. On the basis of phenotypic characteristics and cellular arrangements, Kalina in 1970 named S.faecalis and S.faecium as Enterococcus. In 1984, Schleifer and Kilpper Balz allocated S.faecalis and S.faecium into a separate genus Enterococcus based on DNA-rRNA hybridization studies and 16S rRNA sequencing. [90]

Enterococcus genus include the enterococcal members previously classified with the group D streptococci. 80-90% of human enterococcal infections is caused by the most common isolate E.faecalis.[30,33] E. faecium ranks second and is isolated from 10-15% of infections. Other enterococcal species are infrequently isolated from human infections.

General characteristics of Enterococci:

i) They are gram positive cocci, usually appear oval in shape, arranged in pairs and short chains.

ii) All are facultatively anaerobic.

iii) All are non motile (except E. gallinarum and E. casseliflavus).

iv) Most of them react with the Lancefield group D antisera.

v) They are able to grow at 10°C and 45°C and at pH 9.6.

vi) They survive at the temperature of 60°C for 30 min.

vii) Enterococci are usually alpha or gamma hemolytic on 5% sheep blood agar. Some produce beta hemolysis on human, horse and rabbit blood.

viii) They are capable of growing in media containing 6.5% Nacl.

ix) They are capable of hydrolyzing esculin in the presence of 40% bile salts.

x) They are capable of producing a Leucine aminopeptidase (LAP) and a Pyrrolidonyl arylamidase (PYR) (except for E. cecorum, E. columbae, E. pallens and E. saccharolyticus).

Virulence factors :

1. Cytolysin / hemolysin : It acts on human, rabbit, equine and bovine erythrocytes (but not sheep erythrocytes) and has demonstrated significant toxicity in rabbit endophthalmitis and endocarditis models. The quorum sensing mechanism regulates expression of hemolysin [63].
2. Gelatinase : The gelatinase, a protease that has the capability of hydrolyzing gelatin, casein, collagen, hemoglobin and other peptides. Gelatinase produced by E.faecalis contributed to the pathogenicity of endocarditis in an animal model.[63] gelE coding for gelatinase is involved in biofilm formation.[69]
3. Aggregation substances(AS) : It is a surface bound, plasmid encoded protein that promotes clumping of the organisms to facilitate the plasmid exchange.It facilitates the enterococcal adherence to cultured intestinal and renal epithelial cells and promotes the growth of cardiac vegetations in the rabbit endocarditis model. It is involved in the binding of E. faecalis to neutrophils and cultured intestinal epithelial cells and in subsequent internalization and intracellular survival of these organisms. Agg genes are involved in biofilm

formation.[69] Phagocytosis is significantly resisted by enterococci expressing aggregation substances .

4. Extracellular surface protein (Esp) : It helps the organisms evade antibodies by its ability to be retracted away from the cell surface. Esp gene is involved in biofilm formation. Continued presence of E.faecalis in urinary bladder is enhanced by extracellular surface protein during experimental urinary tract infections.
5. Extracellular superoxide: Most E. faecalis and some E. faecium isolates from bacteremia produce large amounts of extracellular superoxide that may enhance enterococcal virulence in mixed flora abscesses with Bacteroides fragilis.
6. Lipoteichoic acid: It constitutes group D antigen of enterococci and may also contribute to virulence by inducing the production of tumor necrosis factor (TNF) and interferon, leading to modulation of immune response.
7. Coccolysin: 50-65% of E. faecalis strains produce coccolysin, an extracellular metallo endopeptidase, which may play a role in virulence by inactivating endothelin, a vaso peptide.

8. Biofilms : The ability of enterococci to cause device associated infections is enhanced by the production of biofilms. Intravascular catheter – related blood stream infection(CRBSI) by enterococci can be difficult to treat and increases the morbidity. [74]
9. MSCRAMM Ace : Adhesion to collagen of E. faecalis is a collagen binding microbial surface component recognizing adhesive matrix molecule on enterococci. During human infections, there is a expression of adhesion to collagen of E.faecalis.[68]
10. Cell wall carbohydrate and capsular polysaccharide: Clinical isolates of E. faecalis has been identified to express an operon regulating the synthesis of capsular polysaccharide. For prevention of enterococcal infections, antibodies produced against purified carbohydrate may be used.
11. Gls24: It is a general stress protein that has been shown to be important in virulence of E.faecalis in both mouse peritonitis and rat endocarditis models. [44]

Identification of Enterococcal species :

Facklam and Collins proposed physiological five groups to classify enterococcal species based on hydrolysis of arginine and acid production from mannitol and sorbose.[21] Further speciation is based on acid production from sugars like arabinose, sorbitol, raffinose, sucrose, pyruvate, trehalose and motility and pigment production.

Group	Species	Acid from		Arginine hydrolysis
		Mannitol	Sorbose	
I	E. avium	+	+	-
	E. raffinosus			
	E. gilvus			
	E. pallens			
	E. saccharolyticus			

	E. malodoratus			
	E. pseudoavium			
	E. divriesei			
	E. hawaiiensis			
II	E. faecalis	+	-	+
	E. faecium			
	E. gallinarum			
	E. casseliflavus			
	E. mundtii			
	E. hemoperoxidus			
	E. sanguinicola			
	E. ythailandicus			
III	E. dispar	-	-	+
	E. durans			
	E. hirae			
	E. ratti			
	E. villorum			
	E. canintestini			
IV	E. caccae	-	-	-
	E. cecorum			
	E. aquimarinus			
	E. phoeniculicola			
	E. sulfurous			
	E. asini			
	E. silesiacus			
	E. termitis			
V	E. canis	+	-	-
	E. columbae			
	E. moraviensis			
	E. camelliae			
	E.hermanniensis			
	E. italicus			

Clinical presentations of Enterococcal diseases.

Endocarditis and Bacteremia:

Endocarditis and bacteremia are the usual presentations of enterococcal disease. Enterococci are currently one of the leading cause of nosocomial bacteremia. Frequent sources of the bacteremia are usually the genitourinary and gastrointestinal tracts. Intravascular or urinary catheters are the most common source in nosocomial bacteremia and intra abdominal, pelvic, biliary tract, wounds (includes burns patients) and bones have also been documented as sources of the bacteremia. Recent data suggest that E. faecium blood stream infection may have a worse prognosis than E.faecalis because these organisms are much more resistant to antibiotics and are increasingly difficult to treat. The percentage of patients who have endocarditis range from about 1% to 32%. The organisms can affect both native and prosthetic valves and can cause both community and nosocomial associated endocarditis with E.faecalis being recovered much more frequently than E. faecium or other enterococcal species. The disease usually occurs in the settings of damaged heart valves and the mitral and aortic are the valves usually involved, although endocarditis of apparently intact valves has also been reported. Most patients are elderly male with comorbidities. Procedures

associated with the development of enterococcal endocarditis include cystoscopy, caesarean section, prostatectomy, transrectal prostatic biopsy, transjugular intrahepatic portosystemic shunt (TIPS), extracorporeal shock wave lithotripsy, colonoscopy, fiberoptic sigmoidoscopy and liver biopsy. Mortality ranges from 11% to 35% usually due to heart failure or embolization.[45]

Urinary tract infections :

Enterococcal UTIs in young, healthy women without history of urinary tract instrumentation or anatomic abnormalities was first reported in 1906. Enterococcal UTIs are well documented in hospitals and usually associated with indwelling catheters, instrumentation and abnormalities of the genitourinary tract. Enterococci are the third most common organism isolated from catheter associated UTIs with E.faecium, E.faecalis and other enterococcal species accounting for 40%, 25% and 35% of the total enterococcal organisms isolated respectively. Isolation of more than 10^5 colony forming unit (CFU) of enterococcus species from urine may represent colonization and removal of the catheter may suffice to eradicate the presence of the organisms. Recurrent UTIs and previous antibiotic treatment have also been associated

with enterococcal UTIs. Infections appear to be more common in older men and associated prostatitis and epididymitis have been documented. Enterococci can also cause complicated UTIs with the development of pyelonephritis and perinephric abscesses that can lead to bacteremic episodes.

Meningitis :

Enterococci are uncommon causes of meningitis accounting for about 0.3 – 4% of meningitis. The most common species isolated is E.faecalis. Other species associated with meningitis are E.faecium, E.gallinarum, E.avium and E.casseliflavus. Spontaneous and postoperative are the two types of presentation of meningitis. The overall mortality rate approaches 20% and residual sequelae can be seen in about 17% of patients.

Intra-abdominal and pelvic infections :

Enterococci are commensals of the gastrointestinal and genitourinary tracts and are commonly isolated from abdominal and pelvic infections, usually with other gram negative and anaerobic organisms. Enterococci are also capable of producing spontaneous peritonitis and empyema in cirrhotic and chronic

renal failure patients and have also been reported as etiologic agents in peritonitis associated with chronic ambulatory peritoneal dialysis.

Neonatal infections :

Enterococci are part of the normal adult vaginal flora and can be acquired by neonates during delivery. The organisms have been associated with 6% of late onset sepsis, 5% of pneumonia, 9% of surgical site infections, 10% of bacteremia and 17% of urinary tract infections in neonatal units. Enterococcal infections are usually hospital associated and may be polymicrobial. Affected patients usually have a prolonged hospital stay, low birth weight, prior antibiotic therapy and several invasive procedures.

Skin, soft tissue and other infections :

Enterococci have been associated with skin and soft tissue (including wounds) infections. Decubitus and diabetic foot ulcers are the usual lesions associated with the presence of enterococci and they have also been isolated from bone causing osteomyelitis.[45]

BIOFILM FORMATION

Biofilm constitutes a complex community of microorganisms encased in hydrated matrix of exopolymeric substances, proteins, polysaccharides and nucleic acids and attached irreversibly on various biotic(plants, animals, other microbes) and abiotic (minerals, carapaces of dead animals or air water interfaces) surfaces [17]. Complex developmental process involved in biofilm formation are irreversible attachment on a surface, interaction between cell to cell, formation of microcolony, biofilm formation and a three dimensional biofilm structure development.[56] Bacteria in biofilms are extremely difficult to eradicate because they resist phagocytosis and are a cause of chronic infections.[42]

A wide variety of medical devices such as urinary and central venous catheters, cardiac pacemakers, prosthetic heart valves, joint prosthesis, contact lenses and hemodialysis equipment are colonized by biofilm producing bacteria. They are associated with human diseases such as urinary tract infections, burns wound infections, native valve endocarditis, gingivitis, cystic fibrosis and chronic otitis media with effusion.[17]

Factors influenzing formation of biofilm :

i) Nutrient contents like glucose, serum, availability of iron and CO2, osmalarity, pH and temperature of growth medium alters biofilm production.[57]

ii) The association of enterococcal surface protein(Esp) at high glucose concentration in biofilm formation has been reported.[84]

iii) Persistence and colonization of infection within the urinary tract is contributed by enterococcal surface protein of E.faecalis. 93.5% enterococcal surface protein producing E.faecalis produce biofilms on nonliving surfaces and E. faecalis isolates lacking enterococcal surface protein never produced biofilms.[86]

iv) Enhancement of biofilm production in E.faecalis is more in 1% glucose supplemented tryptic soy broth medium than without glucose.[10]

v) The gelatinase, an extracellular zinc metalloprotease is important for biofilm production.

vi) E.faecalis regulator (fsr) the two component quorum – sensing signal transduction system regulates the

expression of gelatinase and serine protease and helps in biofilm formation.[58]

vii) Other genes associated with biofilm formation in E. faecalis are atn (Autolysin), bec (biofilm enhancer in enterococcus), bop (biofilm on plastic surface), dltA (D-alanine lipoteichoic acid) ebpA, B, C (endocarditis and biofilm associated pili), epa (enterococcal polysaccharide antigen), Sal A, B (Secretory antigen like A & B).

Detection of Biofilm formation

Biofilm formation in enterococcus can be detected by qualitative methods such as Congo red agar(CRA) method and Tube method(TM) and quantitative method such as Tissue culture plate (TCP) method. In this study, comparison is done between all the three methods and to find out the most reliable and sensitive method for the detection of biofilm formation.

ANTIMICROBIAL RESISTANCE OF ENTEROCOCCI

Beta lactum resistance:

Formerly, beta lactam antibiotics were the first choice of drugs used in the treatment of enterococcal infections. Relative resistance to beta lactam antibiotics with minimal inhibitory concentration (MIC) of penicillin 10 to 100 times or more those of streptococci is a well described characteristic of enterococci. Penicillins and carbepenems resistance is usually found in clinical isolates of E.faecium and rarely in E. faecalis. The mechanism of resistance in E.faecium involves mutations or overexpression of the PBP5 gene which decrease the affinity of its product for ampicillin. Beta lactam resistance in E. faecalis can be mediated by production of a beta lactamase enzyme.[45]

High level aminoglycoside resistance :

Beta lactam antibiotics are not readily bactericidal for enterococci hence the addition of an aminoglycoside achieves synergistic and bactericidal effect. The aminoglycosides, Gentamicin and Streptomycin are the only two compounds recommended for achieving this synergistic effect in clinical practice. The emergence of HLR to both aminoglycosides was

reported in 1983 and has increased since then in both E. faecalis and E. faecium. High level aminoglycosides resistance(HLAR) is defined by the growth of organism at concentrations of 500mg/L of Gentamicin and 2000mg/L of Streptomycin on brain heart infusion(BHI) agar or 1000mg/L of Streptomycin on brain heart infusion(BHI) broth [47].

The high level resistance to gentamicin is mostly because of the presence of an aminoglycoside modifying enzymes such as 2'phosphotransferase and 6'acetyl transferase which contributes high level resistance to gentamicin and other aminoglycosides except streptomycin. High level resistance to streptomycin can be due to mutations in the 30S ribosomal subunit and to the presence of a streptomycin 6' adenyl transferase [45].

Glycopeptide resistance in Enterococci :

The commonly used glycopeptides in the treatment of enterococcal infections are Vancomycin and Teicoplanin. Vancomycin is a tricyclic glycopeptide antibiotic produced by Streptococcus orientalis.[23] Teicoplanin is obtained from the soil actinomycete Actinoplanes teicomyceticus. It is a mixture of glycopeptide analogues with a basic structure characterized by a

linear heptapeptide, distinct carbohydrates D-mannose and D-glycosamine and an acyl residue that carries various fatty acids. The mechanism of action of these compounds includes inhibition of the cell wall synthesis by the inhibition of the last steps of peptidoglycan synthesis which involves transglycosylation and transpeptidation of the pentapeptide units .

In 1988, Uttley et al were the first to report the isolation of Vancomycin resistant E.faecalis and E.faecium in England.[92] Thereafter the emergence of vancomycin resistance in enterococcal species was reported in Western Europe and in the USA as a therapeutic problem.[90] Transposon, a part of a conjugative plasmid harbouring vancomycin resistance determinants of E.faecium and E.faecalis readily transfers among enterococci and potentially other gram positive bacteria.[27,50] Enterococcal resistance to glycopeptides is the result of alteration of the D-alanyl – D – alanine target to D-alanyl – D – lactate or D-alanyl – D – serine which bind glycopeptides poorly due to the lack of a critical site for hydrogen bonding.

Risk factors associated with Vancomycin Resistant Enterococci:

i) Presence of immunosuppression (haematologic malignancy, bone marrow transplantation).

ii) Presence of co-morbid conditions like diabetes, renal failure, high APACHE (acute physiology and chronic health evaluation) score, malignancy.

iii) Longer duration of hospital stay

iv) Intrahospital transfer to another ward

v) Residence in a long term care facility

vi) Contact with another colonized / infected patient

vii) Invasive procedures

viii) Previous exposure to broad spectrum antibiotics – Cephalosporins, Vancomycin.

ix) Use of enteral tube feeding and sucralfate

x) Exposure to contaminated medical equipment

xi) Exposure to health care personnel who are known VRE patient.

Types of glycopeptide resistance :

1. Van A - It is encoded by Van A gene and is an inducible high level resistance to both Vancomycin (MIC 64-1000µg/ml) and Teicoplanin (MIC 16-512 µg/ml). The altered gene product is "D-alanine – D – lactate". It is mediated by transposon Tn 1546.
2. Van B – encoded by Van B gene. It is an acquired inducible, variable level resistance to Vancomycin (MIC 8 – 1000 µg/ml) but susceptible to Teicoplanin (MIC 0.5 – 1µg/ml). The gene is located in plasmid and mediated by transposons Tn 1547, Tn1549 and Tn 5382. The altered gene product is D-alanine – D – lactate".
3. Van C – encoded by Van C gene located in chromosome and exhibits intrinsic, low level resistance to Vancomycin (MIC 2-32µg/ml) and susceptible to Teicoplanin (MIC 0.5-1 µg/ml) . The gene product is "D- alanine-D- Serine".
4. Van D – encoded by Van D gene, constitutively expressed, chromosome mediated, moderate level resistant to Vancomycin (MIC 64- 128 µg/ml) and susceptible /

resistant to teicoplanin (MIC 4 – 64 µg/ml). The gene product is "D–alanine–D–lactate".

5. Van E, Van G and Van L – encoded by Van E,Van G and Van L Genes located in chromosome results in inducible, intermediate level resistance to Vancomycin (MIC 8 – 32 µg/ml),susceptible to Teicoplanin (MIC 0.5-1µg/ml).[40,47]

Detection of vancomycin resistant enterococci:

1.Antibiotic susceptibility testing by Kirby-bauer disc diffusion method.

2.Detection of vancomycin resistance by using Vancomycin screen agar.

3.Detection of Vancomycin &Teicoplanin Minimum Inhibitory Concentration (MIC) by Broth microdilution method

4.Detection of Vancomycin resistant genotype by Polymerase Chain Reaction(PCR).

This present study was undertaken to detect the biofilm producing Enterococci which were isolated from various clinical materials by three different phenotypic methods such as Tube method,Congo red agar method and Tissue culture plate method and to detect Vancomycin Resistant Enterococci(VRE) by Vancomycin screen agar, Vancomycin and Teicoplanin MIC by Broth microdilution method and genotyping to detect VanA&VanB genes by Polymerase Chain Reaction(PCR) assay.

AIMS AND OBJECTIVES

1. To isolate and speciate Enterococcus from various clinical samples by standard microbiological methods.
2. To detect the biofilm producing strains of Enterococci in clinical isolates by various phenotypic methods.
3. To assess the antibiotic susceptibility patterns of Enterococcal isolates.
4. To study penicillin resistance and high level aminoglycoside resistance by phenotypic method.
5. To study the Vancomycin resistance among the Enterococcal isolates by standard phenotypic method.
6. To assess the prevalence of VanA and VanB genotypes among the Vancomycin Resistant Enterococci(VRE) by Polymerase Chain Reaction (PCR) assay.

REVIEW OF LITERATURE

A prospective study conducted by **Seema sood, Meenakshi Malhotra et al** showed 42.90% of E.faecium and 40% of E.faecalis were the predominant isolates. In blood culture, E.faecium was the commonest isolate. In pus and urine samples ,E.faecalis was predominant[77].Other species such as E.avium, E.mundtii, E.durans, E.dispar, E.raffinosus and E.gallinarum were also isolated[54].In New Delhi study, E.faecium (66%) was the most common isolate in blood samples .The next common isolate was E.faecalis(20%)[35]. A study from Chandigarh reported E.faecalis (55%) followed by E.casseliflavus (24%) and E.faecium (12%) from urinary isolates[83].

BIOFILMS:

According to **Seema Bose, Atindra Krishna Ghosh (2011),**the first recorded observation concerning biofilms was probably given by **Henrichi** in 1933, who observed that water bacteria were not free floating, but that they grew on submerged surfaces[88] . The suggested roles of the biofilms in producing infections are i) detachment of the cells and cause blood stream

and urinary tract infections[18] ii) resistance to host immune system[51] iii) production of endotoxins[29] iv) the generation of resistant organisms[20]. Mechanisms of anti microbial resistance of biofilms are (i) Trapping of antibiotics in the exopolysaccharide matrix[85] ii) Bacteria which are coated with biofilms escape the host immune system[89] iii) Quorum sensing – A cell to cell communication in bacterial biofilms is established through chemical signaling[66][76].

Mandell's principles and practice of infectious diseases(7th edition,Volume 1,page no 23) states that biofilms are proposed to have five developmental phases (i) initial reversible attachment, ii) irreversible attachment, iii) development of biofilm architecture, iv) development of microcolonies v) dispersion of cells from the biofilm[46][81].

Jonathan A.T. Sandoe, Ian R Witherden et al (2003) evaluated that Significant biofilm was produced more by E.faecalis than E. faecium isolates in both Brain Heart Infusion broth and media containing 0.9% Nacl . E.faecalis isolates produced significantly more biofilm in catheter related blood stream infections (CRBSI) than non CRBSI isolates. In saline, E.

faecalis isolates produced high optical density values than in Brain Heart Infusion broth[32].

A study by **Prakash V.P** (2005) conducted in a tertiary care hospital in India reported 44 out of 171 isolates of E. faecalis(26%) and none of the 25 E. faecium(25/171) isolates produced biofilms[65].

CR Kokare, S. Chakraborty et al (2009) stated that Biofilm, a microbial assembly that is strongly attached with a surface and encased in a matrix of Extracellular polymeric substances (EPS). Recently, Quorum sensing which is a Cell to cell signaling has been documented to play a role in cell attachment. The ability of bacterial products to diffuse away from one cell and enter into another cell is responsible for intracellular communication between bacteria[93]. Bacteria in a diffusion – limited environment utilized this method of intracellular signaling. Acyl homoserine lactone (acyl – HSL) is the Quorum sensing molecule. .For analyzing and studying the structure of biofilms,various methods such as Light, Fluorescence, Differential interference contrast (DIC), Transmission electron

(TE), Scanning electron (SE), Atomic force (AF) and Confocal laser scanning microscopy (CLSM) are used [14].

Toledo – Arana, Jaione valle et al (2001) reported that i) the capacity to form biofilms is common among clinical E. faecalis isolates, ii) biofilm formation capacity is restricted to strains harbouring the Enterococcal surface protein (Esp) gene, iii) Esp promotes primary attachment and biofilm formation by E. faecalis on abiotic surfaces [86].

Preeti M.Tendolkar, Arto S.Baghdayan et al (2004) evaluated that Increased amounts of biofilm were produced by two enterococcal surface protein lacking strains FA2-2 and OG1RF after introduction of enterococcal surface protein gene. They noted that N-terminal domain of Enterococcal surface protein (Esp) is needed for biofilm enhancement by E. faecalis because a mutant lacking the N – terminal domain region of enterococcal surface protein(Esp) formed less biofilm than wild type [19].They observed that Enterococcal surface protein (Esp) expression leads to a significant increase in biofilm formation and the contribution of Esp to biofilm formation was found to be most pronounced in the presence of 0.5% or greater glucose. These

results define Esp as a key contributor to the ability of E.faecalis to form biofilms[84] .

Claudia Fedi, Renata O Soares Ana et al (2014) states that virulence factor contribute to enterococcal adherence include biofilm formation, gelatinase (encoded by gel E), enterococcal surface protein (encoded by esp) and aggregation substance (encoded by agg). Besides biofilm formation, gel E hydrolyses collagen, casein and haemoglobin. Aggregation substance mediates the formation of aggregates during conjugation, thus promoting the transference of mobile genetic elements and contributing to pathogenesis. They were also evaluated for the distribution of virulence factors. In this study, 58.3% of isolates harbored agg, 70% harbored esp and 73.3% harbored gel E. Among biofilm formers, 85.3% (174/204) were from noninvasive site (urine) and 58.3% (15/36) were isolated from invasive site. Among the total enterococcal isolates, 81.25% (n=195) isolates produced biofilm. 75.4% (n=147) were high or moderate biofilm producer and 24.6% (n=48) were weak biofilm producer[84].

In **Tomasz et al (2009)** study, biofilm assay and PCR done to evaluate the adherence properties of enterococcal isolates by

detecting the genes esp, agg, ebpA and ebpB. Biofilm assay showed increased adherence in strains when only one of the studied genes was present and even most increased adherence in the case of two genes detected. It was statistically significant for ebpA and ebpB genes and for agg and esp genes ($p<0.05$) [87].

Rezvan moniri, Ahmad Ghasemi et al (2013) evaluated the virulence genes relationship with biofilm formation in enterococcus faecalis isolated from patients with UTI. Biofilm formation assay showed that 16.8% (16/95) of E. faecalis isolates contribute to a strong (OD570 > 0.2) while 83.2% (79/95) exhibit weak (OD570 < 0.2) biofilm formation. There was a significant relationship between the presence of esp (58/95) and acc (6') / aph (2") (65/95) genes and biofilm formation [70].

According to **L.E,Hancock & M.Perego(2004)**, the enzymatic activity of gelatinase is required for its role in biofilm formation. They found that E. faecalis V583 fsr quorum sensing system controls biofilm development by the formation of gelatinase . fsrA, fsrB, fsrC and gel E insertion mutants, got by single cross over recombination had impaired biofilm forming ability.[25]

Giridhara upadhyaya PM, Umapathy BL et al (2009) observed the presence of Enterococcal virulence factors gelatinase, hemolysin and Biofilm among clinical isolates of Enterococcus faecalis. They found that the clinical isolates produced 78(39%), 33(16.5%) and 65 (32.5%) of gelatinase, hemolysin and biofilm formation respectively. Out of 65 biofilm producer, 23(11.5%) were high biofilm producer and 42(21%) were moderate biofilm producer.[69]

Abdul Razak SH ,Hasan et al (2011) evaluated Beta lactamase production, biofilm formation and their antimicrobial susceptibility patterns in 343 clinical samples. The susceptibility of non beta lactamase producing isolates to penicillin were significantly higher than beta lactamase producing isolates ($p<0.001$). The resistance of beta lactamase producing isolates (87.5%) to Vancomycin was significantly higher than that of non beta lactamase producing isolates. The sensitivity pattern of non biofilm former isolates were significantly higher than that of biofilm former isolates [2].

Jayanthi S,M. Ananthasubramanian et al (2008) stated that 40% (10/ 25) isolates were found to be more biofilm

producers on addition of pheromone producing culture E. faecalis FA2-2 strain . One of four (25%) weak biofilm formers and seven of nineteen (35%) moderate biofilm formers developed into strong biofilm producer.[74]

AA Ramadhan, E. Hegedus et al (2013) evaluated that none of the Vancomycin resistant Enterococci (VRE) isolates tested formed a biofilm compared with Vancomycin susceptible enterococci (VSE) which formed biofilms. Three of 11 vancomycin susceptible E. faecium isolates and 6 of 17 vancomycin susceptible E. faecalis isolates showed biofilm formation.[1]

Manpreet kour, I Soni et al (2014) evaluated the most sensitive method of screening for detection of biofilm formation by comparing the three methods such as Tube method(TM), Congo red agar (CRA) method and Tissue culture plate (TCP) method. In Tissue culture plate method, out of 198 enterococcal isolates, 160 (80.8%) were found to be high biofilm formers, 28 (14.1%) were medium and 10 (5.05%) were weak. In Tube method, 87 (43.9%) strong biofilm producers were obtained, 85 (42.9%) were moderate and 26(13.1%) isolates were weak or non

biofilm producers. In Congo red agar(CRA) plate method, out of 198 isolates, 55(27.7%) were strong biofilm producer, 53(26.7%) were moderate and 90 (45.4%) isolates were found to be weak or non biofilm producer. The sensitivity of TCP, TM and CRA methods were 94%, 77% and 38% respectively and specificity of TCP, TM and CRA methods were 83%, 81% and 44% respectively. Therefore this study concluded that TCP method is the most sensitive, accurate and reliable screening method for detection of biofilm formation.[48]

Praharaj Ira, Sistla Sujatha et al(2013) detected the biofilm formation by 3 methods such as Microtitre plate method, Tube method and Congo red agar method. Number of isolates tested positive in Microtitre plate method, Congo red agar method and Tube method were 83 (53%), 75 (48%) and 42 (27%) respectively. The sensitivity and specificity of the Tube method was found to be 61% and 68% as compared to the Microtitre plate method. The sensitivity and specificity of the Congo red agar method was found to be 30% and 77% when compared with the Microtitre plate method for the detection of biofilms. Of the 58 clinical isolates tested for the presence of 'esp' gene, only 28 were

found to be both 'esp' positive and biofilm producers by the Microtitre plate method. Of the 13 'esp' negative strains, seven were found to be biofilm producers while six were negative for biofilm production. 13 out of 32 VRE strains and 70 out of 125 VSE isolates were biofilm producers. No significant difference was observed in the proportion of VRE isolates producing biofilms and the proportion of VSE isolates with biofilm production [64].

VANCOMYCIN RESISTANCE

YA marothi, H Agnihotri et al (2005) stated the two types of antimicrobial resistance in Enterococci – inherent or intrinsic and acquired resistance. (i) Intrinsic resistance is chromosomally mediated and species specific. It is present in all members of the species . Intrinsic antimicrobial resistance by enterococci was expressed to Penicillinase susceptible penicillin (low level), Penicillinase resistant penicillins, Cephalosporins, Lincosamides, Nalidixic acid, low level of Aminoglycoside and low level of Clindamycin. (ii) Acquired resistance is due to either from acquisition of new DNA or mutation in DNA. Examples of acquired resistance include resistance to Penicillin by beta

lactamases, High Level Aminoglycoside Resistance (HLAR), Vancomycin, Tetracycline, Chloramphenicol, high level of Clindamycin and Fluoroquinolone resistance [95].

Louis B. Rice (2001) stated that increased Ampicillin resistance in Enterococci is attributable to either the production of beta lactamase or alterations in the expression or structure of PBP5. Commonly used antibiotics that achieve high gastro intestinal concentrations but are inactive against Enterococci such as the Cephalosporins, Ticarcillin and Vancomycin favour colonization with high levels of VRE [44].

In DK Mendiratta, H Kaur et al 2008) study, high level resistance to both Gentamicin and Streptomycin was observed in 69(46%) isolates by both, high content disc diffusion and agar dilution (MIC) methods. E. faecium isolates (95.5%) showed higher high level aminoglycoside resistance than E. faecalis (37.5%). E.faecium (59.1%) showed higher combined resistance to both the aminoglycosides as compared to E. faecalis (7.8%). In E. faecium High Level Gentamicin Resistance (HLGR) was higher (22.7%) than High Level Streptomycin Resistance (HLSR) (13.6%). HLGR and HLSR strains were

concomitantly resistant to beta lactam antibiotics in both the species. 91.4% of penicillin resistance and 87.2% of ampicillin resistance was found in HLGR while in case of HLSR, it was 93.3% and 88.8% respectively [19].

Robin patel (2003) stated that there was little use of Vancomycin for the treatment of diarrhea caused by Clostridium difficile before the late 1970. Increased use of Vancomycin for the treatment of Methicillin resistance in Staphylococcus aureus and Coagulase negative staphylococci probably contributing to the emergence of Vancomycin resistant enterococci (VRE). In European countries, the use of Avoparcin in animal husbandry was responsible for the emergence of VRE.([72]). **Montecalvo et al** showed that adult patients hospitalized in an oncology unit showed persistent gastrointestinal colonization with VRE for minimum of 7 weeks in the most of the patients studied[53]. A study conducted by **Roghmann et al** in a cancer centre to analyse the VRE colonization showed that 3% had intermittent colonization, 44% had persistent colonization and 33% had clearing of colonization.[73] 8.2% (6 / 73) of VRE colonized paediatric oncology patients[28] and 11.3% (6/ 53) of VRE

colonized liver or kidney transplant patients[60] have been reported to develop clinical VRE infection. In a study comparing the prognosis of patients with Vancomycin sensitive versus Vancomycin resistant enterococcal bacteremia, clinical failure was higher for patients with VRE bacteremia (60%) versus VSE bacteremia(40%)($p<0.001$).

VRE, especially E. faecium has emerged as an important nosocomial pathogen and represents a serious threat to patients with impaired host defences [4]. E. faecium strains as compared to E.faecalis display a higher degree of drug resistance to multiple antibiotics including ampicillin, gentamycin, ciprofloxacin, vancomycin and teicoplanin.[57].

According to **Goodman and Gilman (12th edition, page no 1541)** Transposon ,a part of a conjugative plasmid harbouring Vancomycin resistance determinants in E. faecium and E. faecalis, rendering it readily transferable among enterococci and other gram positive bacteria [22].

According to **Harrison's Textbook of principles of internal medicine (18th Edition Vol I, page no. 1187),** Vancomycin effect is mediated by binding of the antibiotic to

peptidoglycan precursors (UDP – MurNac – pentapeptides) upon their exit from the bacterial cytoplasm. The interaction of vancomycin with the peptidoglycan is specific and involves the last two D- alanine residues of the precursor. The mechanism involves the replacement of the last D-alanine residue of peptidoglycan precursors with D-lactate or D-serine, with consequent high and low level resistance respectively.[26]

In **Sung-ching Pan et al (2012)** study, the incidence and risk factors for VRE was evaluated in surgical ICU patients by collecting surveillance culture- rectal swab after 24 hours of admission, every week after admission and on the day of discharge. Out of 871 patients screened, 34 were found to carry VRE before their admission, 47 acquired VRE during their stay in SICU, five of them developed VRE infections. The incidence of newly acquired VRE during ICU stay was 21.9 per 1000 patient days [82].

Vidyasagar, Nandan et al (2012) compared Kirby bauer disc diffusion method and Vancomycin screen agar method for the detection of Vancomycin resistant enterococci. In this study, out of 45 enterococcal isolates, 57.77% (26) were E. faecalis, 40%

(18) were E. faecium and 3.3% (1) were E. avium. One of the isolates (2%) showed resistance to vancomycin by Kirby – Bauer disc diffusion method. By vancomycin screen agar, 13 isolates showed growth, giving an overall VRE positivity of 29%. In this study, the vancomycin screen agar method was found to be more sensitive in comparison to Kirby-Bauer disc diffusion method for detecting vancomycin resistance in enterococcal isolates.[35]

In **Bahram Fatholahzadeh et al (2006)** study, they observed that out of 120 enterococcal isolates, 7% (8/120) were resistant to vancomycin. Various VRE species were isolated, including 38% of E. faecalis (3/8), 25% of E. faecium (2/8), 25% of E. mundtii (2/8), and 13% of E. raffinosus (1/8). The vancomycin MIC levels among isolates of VRE were quite high (>512µg/ml) indicating high level resistance. Teicoplanin MIC levels of E. faecalis, E. mundtii and E. raffinosus isolates ranged from 8 µg/ml to 32µg/ml and the isolates of E. faecium showed the highest Teicoplainin resistance levels (>64µg/ml).In PCR, 100% (8/8) of VRE species were identified as having the Van A genotype suggesting no diversity in susceptibility genotype among VRE isolates.[7]

According to **Baragundi MC, Sonth SB et al (2010)** study, out of 120 enterococcal isolates, 47.5% (57)of E. faecium,44.16%(53) of E. faecalis, 4.16%(5) of E. mundti, 1.66%(2) of E. durans and 0.83%(1) of E. dispar were isolated. Vancomycin resistance in the range of 0-5% in the enterococcal isolates were shown in many Indian studies. In this study, vancomycin resistance of 7.5%(9) was seen and it showed increasing emergence of vancomycin resistance in the enterococcal isolates .All the nine isolates tested by vancomycin screen agar method and detection of Vancomycin MIC by the macrobroth dilution method showed resistance to vancomycin but Vancomycin resistance in one E. gallinarum isolate could not be detected by the disc diffusion method. High level aminoglycoside resistance (HLAR) was seen in greater than 75% isolates for Gentamicin and in greater than 60% isolates for Streptomycin. Totally 47.18% isolates showed HLAR (both to HLGR+HLSR). **Srujana Mohanty et al** [80] and **Gupta et al** study [24] also showed such high level resistance. The therapeutic options are limited by elimination of the synergy between

aminoglycoside and the beta lactum drugs which is the treatment of choice for enterococcal infections, which is of great concern.[11]

Tripathi A, SK Shukla SK et al (2013) detected vancomycin resistant enterococci by comparing three methods – Chrom ID VRE, PCR and Q-PCR. Out of 145 strains, 80 were VRE and 65 were VSE which were confirmed by disc diffusion and VRE – agar screen methods. 62 E. faecalis and 21 E. faecium were detected as VRE by chrom ID detection method. E. faecium was stained purple and E. faecalis was stained blue or bluish green on Chrom ID detection method. For the detection of MIC to Vancomycin, all VRE strains were grouped into 3 categories: Group I (MIC > 1024 µg/ml), Group II (MIC 512-1024µg/ml) and Group III (MIC < 512 µg/ml) including 55,13,12 isolates in each group respectively. For Teicoplanin, strains were also grouped into two categories. Group I (>512µg/ml) and Group II (MIC 218-256µg/ml). The sensitivity of chrom ID was 100% and specificity was 95.38%.[91] Previous studies suggested that Chrom-ID detection method is better than other chromogenic media and conventional methods for detection of VRE [62,6]. Both the molecular methods such as PCR and Q- PCR were found to be

100% sensitive and specific [91]. PCR and Q -PCR confirmed that all VRE isolates were Van A type. This study concluded that Q - PCR is more rapid and sensitive technique than the other conventional methods for the detection of VRE. [79]

Pedro Alves d'Azevedo et al (2009) identified enterococcal species and vancomycin – resistant genes by rapid methods such as VREBAC medium and multiplex PCR. VREBAC medium contains 3 types of media. (a) Medium I contained agar azide and the selective antimicrobials aztreonam, polymyxin B and amphotericin B. (b)Medium II contained vancomycin 6 µg/ml.(c) Medium III had the same medium II antimicrobial and a chromogenic marker. During the out break, all enterococcal isolates (100%) grew in the medium I. 78.3% of positive growth was shown by medium II which contained vancomycin 6µg/ml. 71.1% isolates was identified by the chromogenic and selective medium. Medium III was able to select and distinguish between vancomycin resistant E. faecalis and vancomycin resistant E. faecium [41]. The multiplex PCR identified 48.8% of E.faecium and 51.2% of E. faecalis and they were all positive for the gene Van A [61].

Server YAGCI, Serife ALTUN et al (2013) evaluated Cepheid Gene Xpert system for the rapid detection of vancomycin resistant enterococci. The gene Xpert system(Cepheid, Sunnyvale, CA, USA) merges automated nucleic acid sample preparation, amplification and real time detection of enterococcus DNA in a disposable, macro / microfluidic catridge using the GeneXpert, Dx system instrument and generally provides results in less than 1 hr [13]. The number of positive samples by Gene X pert Van A / Van B method was 7.3% (33) among the 454 rectal specimens. It detected 4.9% (22) samples that showed only Van A harboring VRE, 2.2%(10) with only Van B harboring VRE and 0.2%(1) sample with both Van A and Van B harboring VRE [77].

TREATMENT OF VANCOMYCIN RESISTANT ENTEROCOCCI

1.Quinupristin-Dalfopristin:

It is a parenteral semisynthetic antibiotic combination of streptogramin A (dalfopristin) and type B (quinupristin). It inhibits protein synthesis by targeting the bacterial 50s ribosome.[5] Resistance can develop through modification of the target binding site, enzymatic inactivation and/or efflux. Arthralgias and myalgias are the most common adverse effects which can be debilitating and have limited widespread use of the agent.[43,94] E. faecium is susceptible whereas E. faecalis and other enterococcus species are intrinsically resistant.

2. Linezolid:

It belongs to oxazolidinones group. Its mechanism of action is by inhibition of ribosomal protein synthesis, at a different site than other agents . It is active against E. faecium, E. faecalis, E. casseliflavus and E. gallinarum. It can be administered orally or intravenously. The major adverse effect thrombocytopenia, may limit its use.

3. **Daptomycin:**

It is a cyclic lipopeptide fermentation product of Streptomyces roseosporus. It rapidly kills gram positive bacteria by disrupting multiple aspects of bacterial membrane function.[67] Because of high failure rate, Daptomycin should not be used to treat pneumonia [31]. It was approved by FDA for the treatment of complicated skin and soft tissue infections.[47]

4. **Tigecycline :**

It is a broad spectrum glycylcycline antimicrobial agent. This new tetracycline derivative has activity against both gram positive and gram negative aerobic and anaerobic bacteria. This compound has in vitro activity against VRE. It is used for the treatment of abdominal and complicated skin and soft tissue infections.[5]

5. **Other anti enterococcal agents:**

Nitrofurantoin has been used to treat UTIs caused by VRE. Quinolones have been used as a combination therapy for the treatment of enterococcal infections. Tetracycline and chloramphenicol have been used in combination therapy. The drug Fosfomycin tromethamine has activity against enterococcal

urinary tract infections. Future options for the treatment of Vancomycin resistant enterococcal infections may include novel glycopeptides like Mannopeptimycins and Dalbavancin [5].

MATERIALS AND METHODS

The present study was conducted in Government Rajaji Hospital, Madurai Medical College. The study period was from August 2013 to July 2014. Ethical committee clearance from the institution was obtained and informed written consent was received from the patients before collecting the specimens. A total of 482 clinical samples were collected from the patients admitted in various wards of Government Rajaji Hospital, Madurai.

Source and Sample size :

Urine samples, blood samples, pus and wound swab samples were collected from 482 patients admitted at Govt. Rajaji Hospital during the study period.

Inclusion Criteria :

i) All age groups and both sexes were included.

ii) Patients admitted to various wards (ICU, CCU, Surgery, Medicine, Pediatrics, Urology) with signs and symptoms suggestive of impending infections such as post operative wound infection, wound infection following burns ,pyrexia of unknown origin, urinary tract infection,

meningitis, endocarditis, intra abdominal abscesses and septicemia were included in this study.

iii) Exclusion criteria

i) All other wound infections

ii) Abscesses other than intra abdominal abscess

Collection of specimens:

i) Collection of blood samples:

Blood samples were collected by sterile aseptic procedures. The hands were kept clean and dry, sterile gloves were worn. The skin over the venepuncture site was disinfected by applying 70% alcohol followed by 1% iodine for atleast 1 minute and allowed to dry. 5 ml of blood was withdrawn without touching the skin to avoid recontamination of the venepuncture site. About 5ml of blood was inoculated into 50ml of brain heart infusion broth [55].

ii) Collection of urine samples :

The female patients were instructed to hold the labia apart and male patient to retract the foreskin, after washing the genitalia with soap and water. After several ml of urine have been passed, midstream portion of the urine was collected into a sterile, leak proof, wide mouthed ,screw capped container .The collected urine

was transported to the laboratory within 30 minutes of collection. If there is a delay of 1-3hours, refrigerate the specimen at 4°C [55].

iii) Collection of wound swab samples :

Two sterile cotton swabs were used to collect the samples from the patients, which can be used for direct smear examination and culture respectively. The swabs were transported in sterile test tubes to the laboratory immediately.[55]

iv) Collection of pus samples :

The wound site was decontaminated with 70% ethyl or isopropyl alcohol, after which the wound was washed well with sterile saline and dried. Using sterile syringe and needle, pus was aspirated and then the specimen was transported in a sterile container.[55]

Processing of Samples :

The collected samples were properly labeled with Name, age, sex and IP/OP no. of the patient, date and time of collection of the sample and brought to the laboratory and processed immediately.

Blood samples :

Brain heart infusion (BHI) broths were incubated at 37°C for 18-24 hrs after which the broths were examined for turbidity and subcultured onto Nutrient agar, MacConkey agar and Blood agar for isolation of organisms.

Urine samples :

Wet mount and gram staining were performed from uncentrifuged urine. After that urine samples were inoculated onto the Nutrient agar, MacConkey agar and Blood agar. Before inoculation, urine was mixed thoroughly and the cap of the container was removed. The calibrated loop was inserted vertically into the urine container. The centre of the plate was touched with the loop and the inoculation was spread in a line across the diameter of the plate. Without flaming or reentering urine, the loop was drawn across the entire plate, the first inoculation was crossed numerous times to produce isolated colonies. The plates were incubated at 37°C for 18-24 hours. A colony count of $>10^5$ CFU/ml was taken as indicative of positive culture.

Wound swab and pus samples :

Direct gram stain was done from samples followed by inoculation onto Nutrient, MacConkey and Blood agar.

Culture identification :

After 24 hrs of incubation at 37°C, plates were examined for the presence of growth and the organisms were identified by colony morphology on solid media, gram staining, biochemical reactions and other identification tests.

Identification of Enterococcus species:

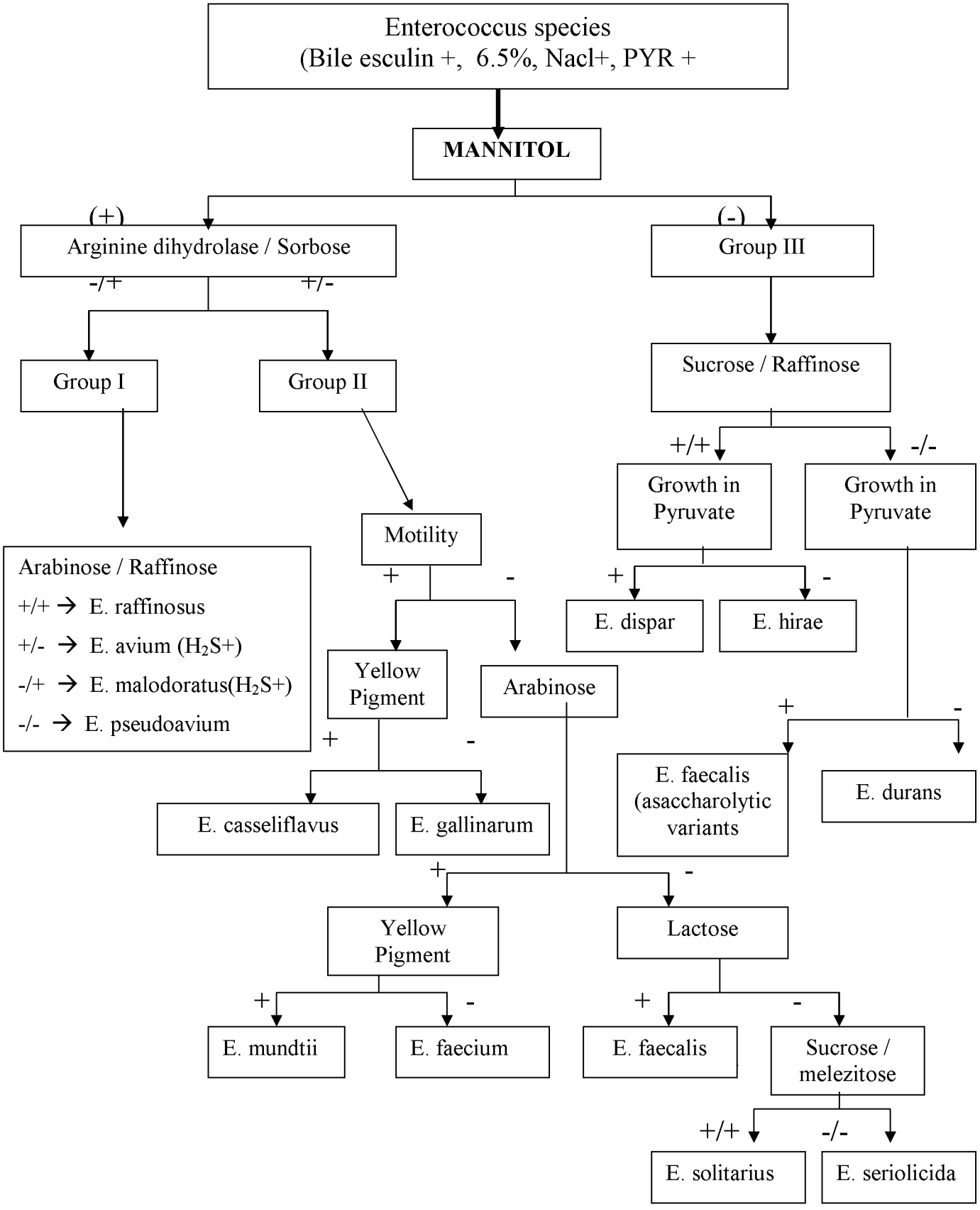
1. Nutrient agar : Tiny, opaque colonies
2. MacConkey agar : Tiny, magenta pink colonies
3. Blood Agar : alpha / beta / non hemolytic colonies
4. Gram staining : Gram positive cocci in pairs and short chains.
5. Catalase test : Negative
6. Bile esculin test – hydrolyze esculin in the presence of 40% bile – blackening of the medium.
7. 0.04% Tellurite agar produces black coloured colonies.

8. Heat tolerance test – Growth can be detected before and after heating at a temperature of 60° C for 30 minutes in a water bath.
9. Salt tolerance test – Growth in 6.5% sodium chloride (Nacl) broth.
10. Mannitol motility medium : Non motile, ferment mannitol by producing acid only.
11. Arginine dihydrolase test – Hydrolyses arginine – produces deep purple colouration after initial colour change to yellow.
12. Carbohydrate (1%) fermentation test – differs in various species.

Species identification of Enterococcus

Based on biochemical tests such as acid produced from arabinose, mannitol, pyruvate, Raffinose, Sorbitol, Sucrose, and Sorbose, NH3 produced from arginine, esculin hydrolysis, ability to grow in 6.5% Nacl broth, presence of Pyrrolidonyl peptidase (PYRase) and Leucine aminopeptidase (LAP), motility and pigment production, species of enterococci were identified. E. faecalis was the most commonly encountered species followed by E. faecium.

FACKLAM AND COLLIN FLOW CHART FOR IDENTIFICATION OF ENTEROCOCCUS SPECIES



Identification Tests

1. Gram staining :

Smear was prepared from the test organism taken from the agar plate, air dried and heat fixed. The smear was flooded with 0.5% methyl violet and washed with water after 1 minute. Then flooded the smear with Gram's iodine and washed with water after 1 minute. Then the smear was decolourised with few drops of acetone and immediately washed with water. Dilute carbol fuchsin, the counterstain was added to the smear and washed with water after 1 minute, the smear was blotted dry and seen under oil immersion objective. Violet colored, spherical cocci arranged in pairs and short chains were identified as Gram positive cocci, presumably Enterococci.

2.Catalase test (Tube method)

Procedure:

2-3ml of 3% hydrogen peroxide was taken in a clean test tube. Few colonies of the test organism were taken from the nutrient agar plate with a sterile glass rod or wooden stick and introduced into the test tube containing H_2O_2.

Interpretation :

Catalase producing organisms split hydrogen peroxide into water and oxygen and produce brisk effervescence due to the release of oxygen. Organisms lacking catalase will not split hydrogen peroxide and will not produce brisk effervescence. The genus Enterococci were catalase negative.

3. Bile Esculin Test :

Procedure:

2-3 morphologically similar test organism were taken with a sterile inoculating wire or loop and streaked onto the surface of a bile esculin agar plate. The plates were incubated at 35°C for 24-48 hours in an incubator.[38]

Interpretation :

Diffuse blackening of the medium or production of black haloes around isolated colonies is considered positive bile esculin test. In the presence of 40% bile, esculin, a glycoside coumarin derivative is hydrolysed into glucose and esculetine which inturn reacts with ferric ions to form a black diffusible complex. All enterococci were bile esculin positive.

4. Heat tolerance test :

Procedure : Test organism from 18-24 hour culture was inoculated into brain heart infusion (BHI) broth and incubated at 60°C for 30 minutes in a water bath. The broth was subcultured onto blood agar and MacConkey agar before incubation and after incubation. ATCC E. faecalis 29212 is used as a positive control.[96]

Interpretation :

The ATCC control strain showed growth both before and after heating the broth at 60°C for 30 minutes. The isolates showing growth before and after heating were taken as heat tolerant enterococcal species.

5.**Salt tolerance test :**

Procedure :

2 to 3 colonies from an 18-24 hours culture of suspected enterococcal isolates were inoculated into a tube containing nutrient broth with 6.5% Nacl. The broth contains bromocresol purple as the indicator for acid production. The tubes were incubated at 35 – 37°C in ambient air for 48 hours. Enterococus faecalis ATCC 29212 is used as a positive control.[9]

Interpretation :

The broth showed visible turbidity with or without a color change from purple to yellow indicates positive reaction. All the enterococcal isolates were salt tolerant and grew in the presence of 6.5%Nacl.

6.Arginine dihydrolysis Test :

Procedure:

The enterococcal isolates were inoculated into a tube of Moeller's decarboxylase broth containing arginine and a control tube containing Moeller's decarboxylase base broth (without arginine) and overlaid both the tubes with sterile mineral oil. The tubes were incubated at 37°C for 4 days. The tubes were examined at 24, 48, 72 and 96 hours.[38]

Interpretation :

Development of deep purple colour due to alkalinization after an initial change to yellow colour indicates positive reaction. Development of persistant yellow colour indicates negative reaction. Both Enterococcus faecalis and Enterococcus faecium were arginine dihydrolysis test positive.

7.Mannitol Motility Test :

Procedure :

The mannitol motility medium was stab inoculated with the test organism and positive and negative control. The tubes were incubated at 37°C for 24 hours. Positive control used was Escherichia coli ATCC 25922 and negative control used was Staphylococcus aureus ATCC 25923 [8].

Interpretation:

Motile organism was evident by a diffuse zone of growth extending out from the line of inoculation. Non motile organisms remained at the site of inoculation. Mannitol fermenting organisms changed from red color to yellow color due to acid production. Enterococcus faecalis and Enterococcus faecium were mannitol fermenting and nonmotile.

8.Carbohydrate Fermentation test :

Procedure :

The enterococcal isolates were inoculated into carbohydrate fermentation media containing 1% carbohydrate such as pyruvate, arabinose, sorbitol, sucrose and raffinose. The tubes were

incubated at 37°C for 24 hours. Bromothymol blue is used as an indicator.

Interpretation :

Acid production from carbohydrate was indicated by the color change from blue to yellow.

Detection of Biofilm production :

`Detection of biofilm production in Enterococcal isolates was done by the following methods.

1. Congo red agar method (CRA)
2. Tube method(TM)
3. Tissue culture plate (TCP) method

1. Congo red agar method :

It is a qualitative method used for the detection of biofilm formation. The medium used was Congo red agar (CRA) medium.

Procedure :

The test organisms were inoculated in CRA medium and kept for incubation at 37°C for 24 hr aerobically.[49]

Intrepretation :

Biofilm producer	Colony Morphology
High	Colonies with black colour and a dry crystalline consistency
Moderate	Darkening of the colonies without dry crystalline consistency
Weak / Non Biofilm producers	Pink coloured colonies

2. Tube method :

A qualitative method for detection of biofilm production

Procedure :

1. The test organisms were inoculated in 10ml of trypticase soy broth taken in the sterile test tubes. The tubes were kept for overnight incubation at 37°C .
2. Then the tubes were decanted and by using phosphate buffer saline (pH 7.3), the tubes were washed and then allowed to dry.
3. By using 0.1% safranin, the tubes were stained and deionized water was used to remove excess stain.

4. Tubes were kept in inverted position and allowed to dry. The control strains were included in the test and according to the results the scoring was done.[49]

Interpretation :

Biofilm production :

The wall and the bottom of the tube were lined by a visible film. The amount of biofilm formed was scored as 1–weak / none, 2 – moderate, 3 – strong.

3. Tissue culture plate method :

This is a quantitative method for biofilm detection.

Procedure :

1. The test organisms were inoculated in 10ml of trypticase soy broth and kept for overnight incubation.
2. A dilutions of 1 : 100 was done for the cultures by using fresh broth. 200µl of the diluted cultures was added into individual wells of sterile 96 well flat bottom polystyrene tissue culture plate and then incubated along with positive and negative control. (Biofilm producer was Enterococcus faecalis ATCC 29212 and biofilm nonproducer was Staphylococcus aureus ATCC 25923).

3. Gentle tapping was done to remove the contents of the well.
4. Washing of the wells was done with 0.2ml of phosphate buffer saline (pH 7.2) and then wells were washed four times to remove the free floating bacteria.
5. After washing, 2% sodium acetate was used to fix adherent bacteria in the wells and by using 0.1% crystal violet, the wells were stained and deionised water was used to remove excess and then allowed to dry.
6. Reading was taken at wavelength 490nm by micro ELISA autoreader. As the bacteria forms biofilm and adheres to the wells, these optical density values were taken as an index of bacterial adherence to the wells [49].

Interpretation

Mean OD values	Biofilm production
< 0.1	Non / weak
0.1 – 0.2	Moderate
> 0.2	High

OD cut off value = average of negative control +

3 x Standard deviation of negative control

The sensitivity, specificity, positive predictive value(PPV) and negative predictive value(NPV) of each test were calculated by using true positive, true negative, false positive and false negative values obtained in all the three methods.

Antibiotics susceptibility Testing

1. Preparation of inoculum :

Morphologically similar 4 to 5 isolated colonies of Enterococci were taken from 24 hrs culture plate with the help of a sterile loop and transferred to a test tube containing sterile peptone water and incubated at 37° C for 4 hours. Then the turbidity was adjusted to 0.5 McFarland turbidity standards by using Wickerham chart. This inoculum was used for antibiotic susceptibility testing.

Antibiogram by Kirby – Bauer disc diffusion method :

According to CLSI guidelines (CLSI document M02&M07) ,the antibiotic susceptibility test was performed by using the Kirby – Bauer disc diffusion technique. Sheep blood agar was used for antibiotic susceptibility test. A sterile swab was dipped in the standardized inoculum and the excess fluid was squeezed out by

pressing on the side of the test tube and it was streaked on the surface of the agar three times, turning the plate at 60° each time to produce a lawn culture of the organism. Then antibiotic discs were placed within 15 minutes of inoculation. The inoculated plates were incubated aerobically at 37°C overnight. Zone size was interpreted using control strain Enterococcus faecium BM4147 under reflected light except for Vancomycin which should be read through transmitted light. The interpretation as susceptible, intermediate and resistant was done according to the CLSI guidelines.[15]

Antimicrobial Drug	S	I	R
Ampicillin 10µg	≥17	-	≤16
Ciprofloxacin 5µg	≥21	16-20	≤15
Doxycycline 30µg	≥16	13-15	≤12
Vancomycin 30µg	≥17	15-16	≤14
Teicoplanin 30µg	≥14	11-13	≤10
High level gentamicin (HLG)120µg	≥10	7-9	≤6
High level streptomycin (HLS)300µg	≥10	7-9	≤6
Linezolid 30µg	≥23	21-22	≤20
Nitrofurantoin 300µg	≥17	15-16	≤14

Zone size (mm) S- Sensitive, I – Intermediate, R – Resistant

Screening test for high level aminoglycoside resistance (HLAR)

The enterococcal isolates were screened for high level resistance to aminoglycosides using the high level gentamicin (HLG) – 120 µg and high level streptomycin (HLS) 300 µg on

sheep blood agar by standard disc diffusion method as described above. Bacterial suspension equal to 0.5 McFarland standard was used and incubated at 37°C overnight. The test also included E. faecalis ATCC 29212 as negative control and E. faecium BM 4147 as positive control.

Detection of Vancomycin resistance :

Vancomycin screen agar that is brain heart infusion (BHI) agar containing 6 µg/ml of Vancomycin was used for the presumptive identification of vancomycin resistance. Spot inoculation of 10µl of 0.5 McFarland turbidity standard bacterial suspension along with positive and negative control strains was done on to the agar surface and the plates were incubated for 24hrs aerobically at 37°C. Growth of greater than 1 colony indicates presumptive vancomycin resistance. It should be confirmed by determining the minimum inhibitory concentration (MIC) for vancomycin and teicoplanin. This test included E. faecalis ATCC 29212 as negative control and E. faecium BM 4147 as positive control.

Vancomycin MIC by Broth microdilution method :

As per the CLSI recommendations, the minimum inhibitory concentration of vancomycin and teicoplanin for the Enterococcal isolates grown on Vancomycin screen agar were done.

Preparation of inoculum :

Bacterial inoculum is prepared by direct colony suspension method and standardized with 0.5 McFarland standard. 1 in 100 dilution of this inoculum is prepared by adding 0.01ml of the inoculum to 0.9ml of Cation Adjusted Muller Hinton Broth (CAMHB). 10 µl of this diluted suspension is equivalent to the final recommended inoculum of 5 x 10^4 CFU/ml and it is dispensed per well.[16]

Preparation of Drug concentrations of Vancomycin :

Stock solution of the drug is prepared as recommended in CLSI document on dilution testing. The vancomycin drug is dissolved in distilled water and the master dilution is prepared by diluting the required amount of drug in CAMHB. Serial doubling dilution of the master dilution was performed in CAMHB such as 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512,1024 µg/ml.

100µl of serial doubling dilution of Vancomycin along with 10µl of bacterial suspension was dispensed in the corresponding wells with a growth control (well containing CAMHB and bacterial inoculum without Vancomycin) and sterility control (well containing CAMHB only) and the microtitre plate was incubated for 24 hrs at 37°C in an ambient air. The test included E. faecalis ATCC 29212 as negative control and E. faecium BM 4147 as positive control.[12]

Minimum inhibitory concentration (MIC) is the minimum concentration of the drug showing complete inhibition of bacterial growth visible to the naked eye. Results were interpreted after reading the MIC of control strains. The MIC range of E. faecalis ATCC 29212 should fall within the range of 1-4µg/ml. The MIC of other isolates were interpreted as per CLSI guidelines.

Minimum inhibitory concentration for Teicoplanin:

The teicoplanin MIC was also tested in the same method as described above.The concentrations of Teicoplanin prepared were 0.125,0.5,1,2,4,8,16,32,64,128µg/ml by dissolving the teicoplanin in Cation Adjusted Muller Hinton Broth (CAMHB) as described in the CLSI guidelines. The bacterial inoculum was prepared by

direct colony suspension method and standardized with 0.5 McFarland turbidity standard.1 in 100 dilution of the bacterial inoculum was prepared in the same way as described above.100µl of each drug concentration was dispensed into the microtitre plate well leaving the growth control well and 10µl of the bacterial inoculums was dispensed into all the wells except sterility control well. The microtitre plate was incubated at 37°C for 24 hours. The MIC results were interpreted by noting the minimum concentration of the drug showing complete inhibition of the growth of the organism in the same way as described for Vancomycin [12].

Molecular method for the detection of Vancomycin resistant Enterococci :

Polymerase chain reaction (PCR) assay was done for the detection of Vancomycin resistance genes in Enterococci especially in E. faecalis and E. faecium by the PCR kit procured from Helini Biomolecules, Chennai.

DNA was extracted by using PureFast R Bacterial genomic DNA purification kit.2X PCR Master mix contained 2U of Taq

polymerase, 10X Taq reaction buffer,2mM Mgcl2,1µl of 10mM dNTPs mix and PCR additives. Agarose gel electrophoresis was performed with agarose, 50X TAE buffer,6X gel loading buffer and Ethidium bromide .

Procedure :

About 1.5 ml of overnight bacterial culture was taken and centrifuged at 10,000 rpm for 5 min. supernatant was discarded.180µl of Lysozyme digestion buffer was added to the pellet. Then 20µl of Lysozyme was added .It was incubated at 37°C for 15 minutes.

400µl of Lysis buffer and 20 µl of proteinase K(10mg/ml) was added and mixed well. Again it was incubated at 56ºC for 10 minutes in a water bath. Then 300µl of isopropanol was added and mixed well by inverting many times. By using pipette, the entire sample volume was transferred to pure fast spin column and centrifuged at 10,000 rpm for 1 minute. Flow through was discarded and 500µl of wash buffer-1 was added. It was centrifuged at 10,000rpm for 1minute. Flow through was discarded and 500µl of wash buffer-2 was added. Then it was centrifuged at 10,000rpm for 1 minute. Washing was repeated one

more time. The flow through was discarded and the column was centrifuged for additional 2 minutes to remove any residual ethanol.

100µl of Elution Buffer was added to elute DNA and centrifuged for 1 minute. Quality and Quantity of extracted DNA is checked by loading in 1% agarose gel. 5µl of extracted DNA is used for PCR amplification.

PRIMER:

Van A Primers

Forward primer:5'-TGCGCGGAATGGGAAAACGACA-3'

Reverse primer:5'-CAGCCCGAAACAGCCTGCTCAA-3'

PCR Product size:473bp

Van B Primers

Forward Primer:5'-TCTTTGTGAAGCCGGCACGGTC-3'

Reverse Primer:5'-AGCCGACCTCACAGCCCGAAAT-3'

PCR Product size:147bp

PCR amplification :

The PCR reactant mixture for each sample is prepared by adding 10µl of PCR master mix, 5µl of primer mix and 5µl of purified DNA of each sample to a total final volume of 20µl.

PCR Procedure:

20µl of the PCR reactant mixture was mixed gently, spin down briefly and placed into PCR machine. It was programmed as follows:

Initial Denaturation: 94ºC for 3 min

Denaturation: 94ºC for 30sec
Annealing: 58ºC for 30sec } 35 cycles
Extension: 72ºC for 30sec

Final extension:72º C for 5 min

Loading:

2% agarose gel was prepared by mixing 2gm of agarose in 100ml of 1XTAE buffer. 8µl 6X Gel loading dye was added to each PCR vial and 5µl of PCR sample was loaded. After that run electrophoresis at 50V till the dye reaches three fourth distances. The bands were observed in UV transilluminator.

Agarose gel electrophoresis:

2% agarose was prepared by the addition of 2gm agarose in 100ml of 1X TAE buffer (melted using microoven).When the agarose gel temperature was around 60°C,5µl of Ethidium bromide

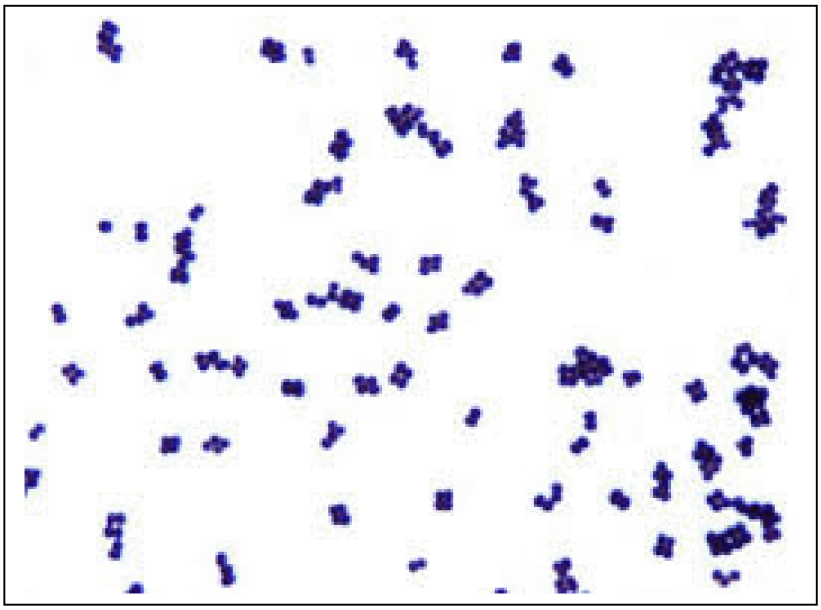
was added. Warm agarose solution was poured slowly into the gel platform. The gel set was kept undisturbed till the agarose solidifies. 1X TAE buffer was poured into submarine gel tank. The gel platform was placed carefully into tank. The tank buffer level was maintained 0.5cm above than the gel.PCR Samples were loaded after mixed with gel loading dye along with 10µl of 100bp DNA Ladder. (100bp,200bp, 300bp,400bp, 500bp, 600bp, 700bp, 800bp, 900bp, 1000bp). Then electrophoresis was run at 50 V till the dye reaches three fourth distance of the gel. Gel was viewed in UV Transilluminator and observed the bands pattern.

INTERPRETATION:

The presence of VanA and VanB genes were indicated by the amplification of 473bp and 147 bp PCR product from the clinical isolates respectively.

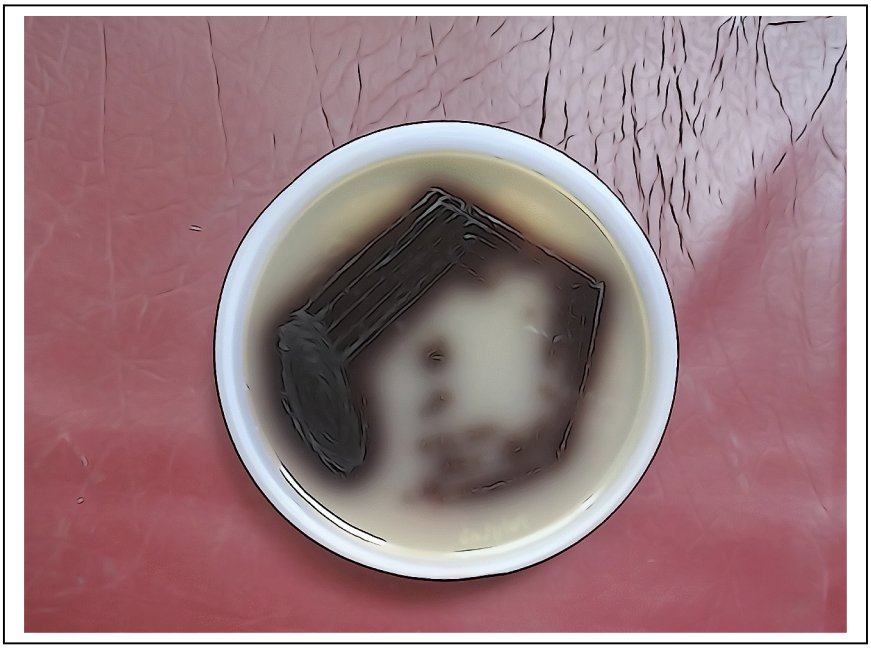
The prevalence of Vancomycin resistance among enterococcal isolates was evaluated by comparing phenotypic classification of vancomycin resistance by Vancomycin and Teicoplanin MIC by Microbroth dilution method and genotypic detection of Van A and Van B resistance genes by Polymerase Chain Reaction(PCR) assay.

GRAM POSITIVE COCCI IN PAIRS AND SHORT CHAINS

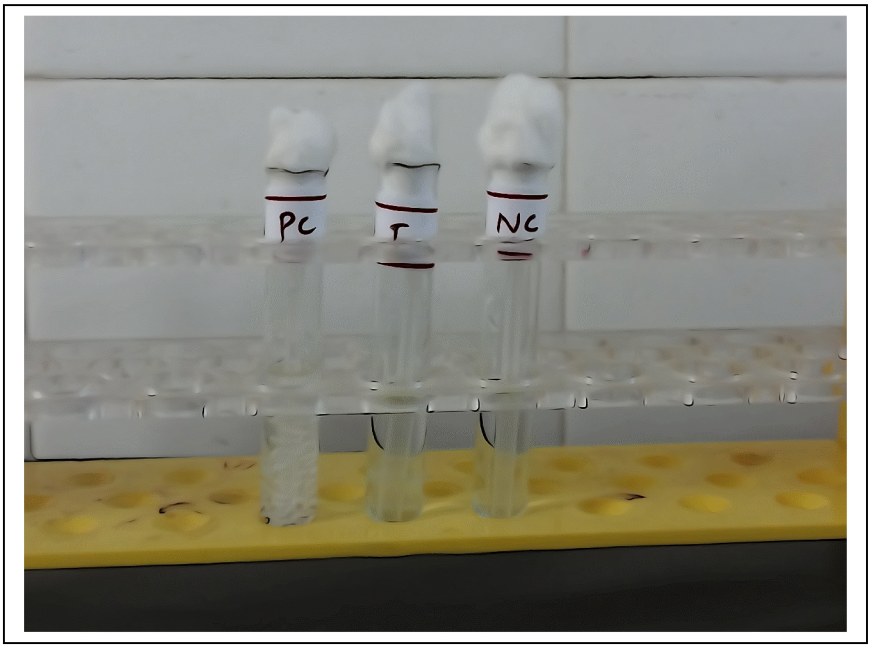


BAP SHOWING ALPHA HEMOLYTIC COLONIES

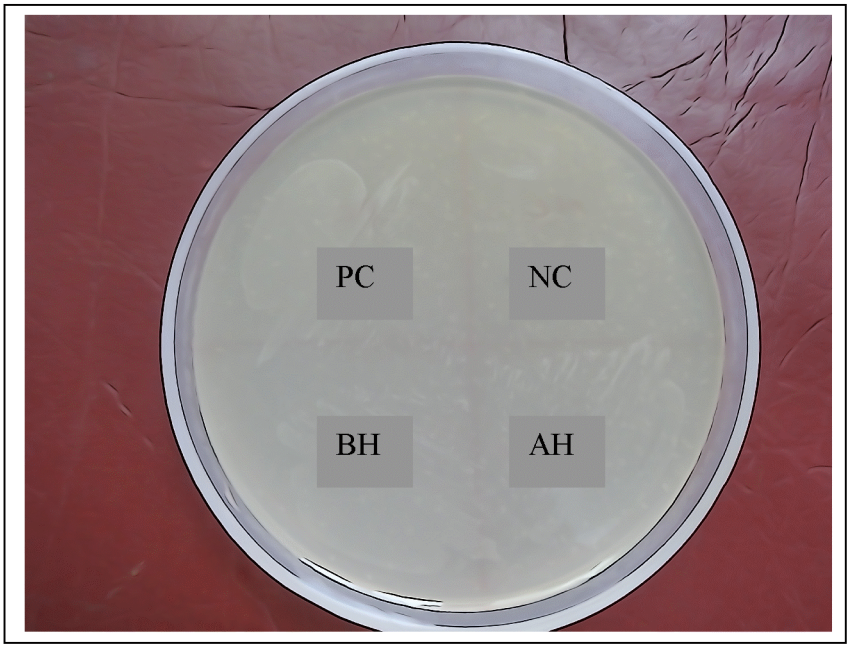
BILE ESCULIN AGAR –BLACKISH DISCOLORATION OF MEDIUM BY ENTEROCOCCUS SPECIES



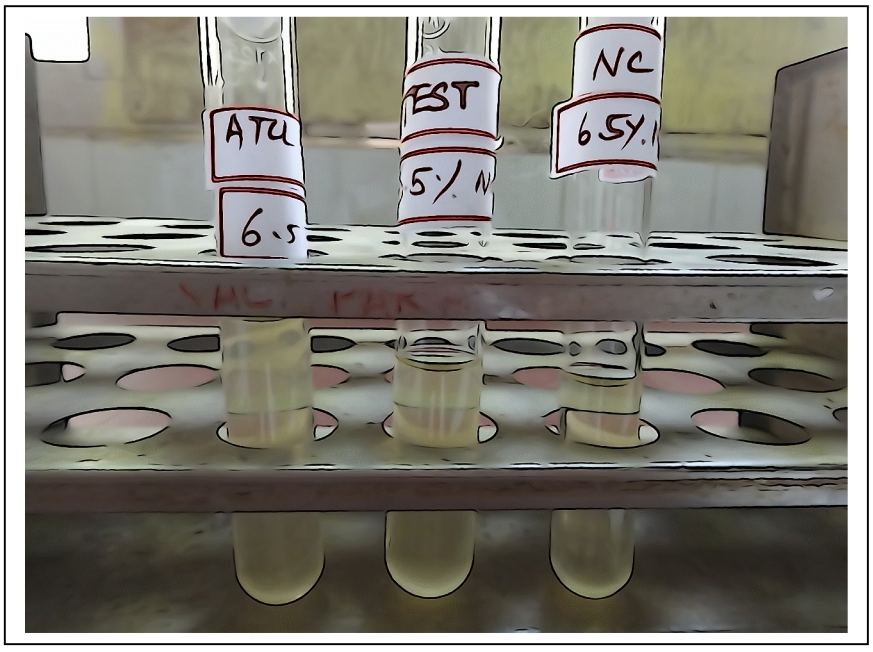
BIO CHEMICAL TEST
CATALASE TEST SHOWING NEGATIVE CATALASE REACTION



HEAT TOLERANCE TEST

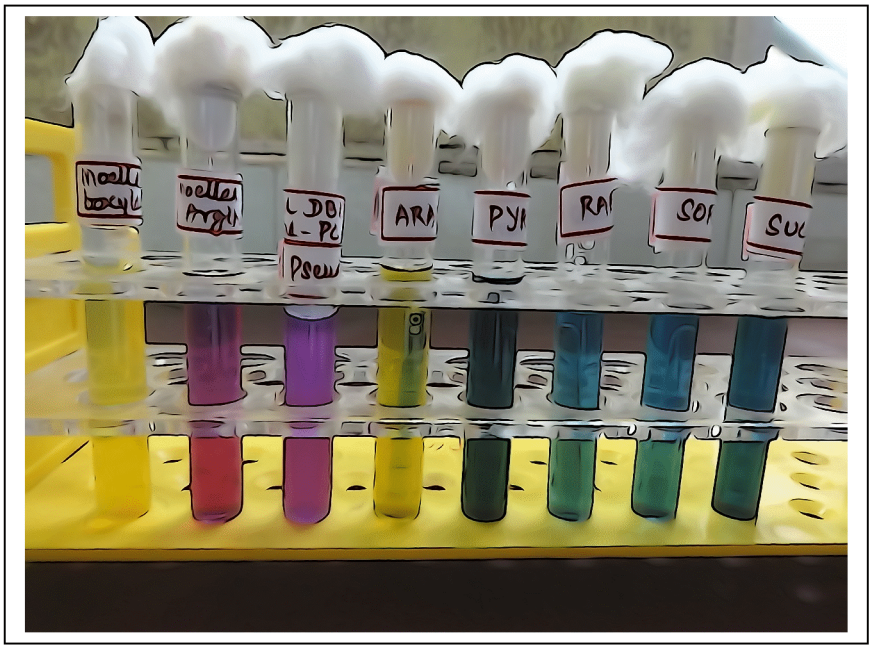


SALT TOLERANCE TEST



MANNITOL MOTILITY TEST
Lt-Mn+,Motile Rt-Mn+,Non motile

BIOCHEMICAL REACTIONS OF E. faecium
Arg+,Ara+,Pyr-,Raf-,Sor-,Suc-

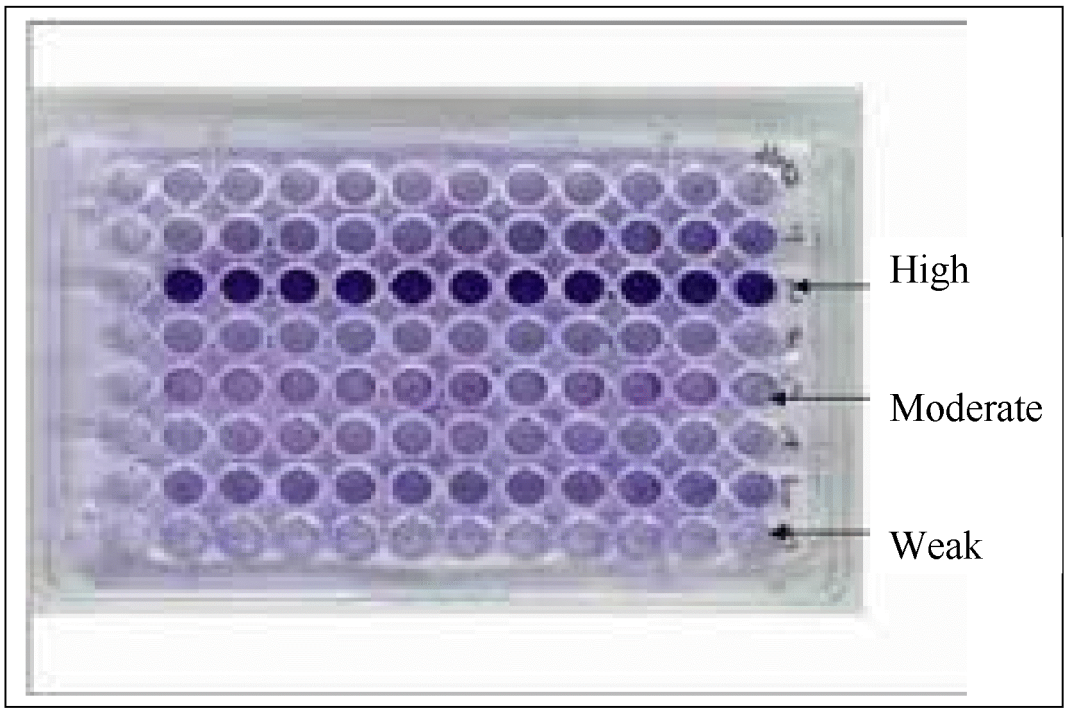


BIOCHEMICAL REACTIONS OF E. faecalis
Arg+,Ara-,Pyr+,Raf-,Sor+,Suc-

BIOFILM PRODUCTION
CONGO RED AGAR-BIOFILM
POSITIVE NEGATIVE

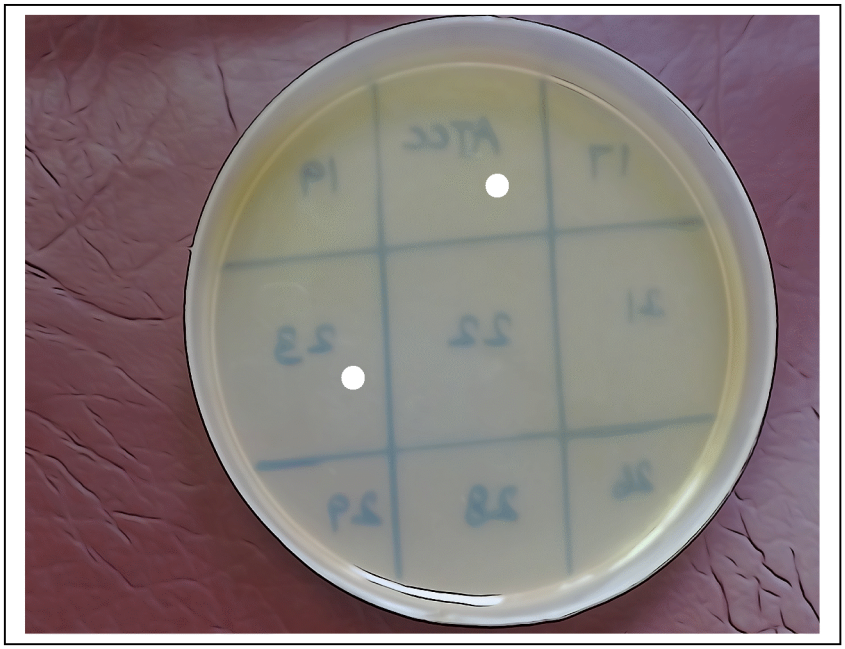
TUBE METHOD

TISSUE CULTURE PLATE METHOD

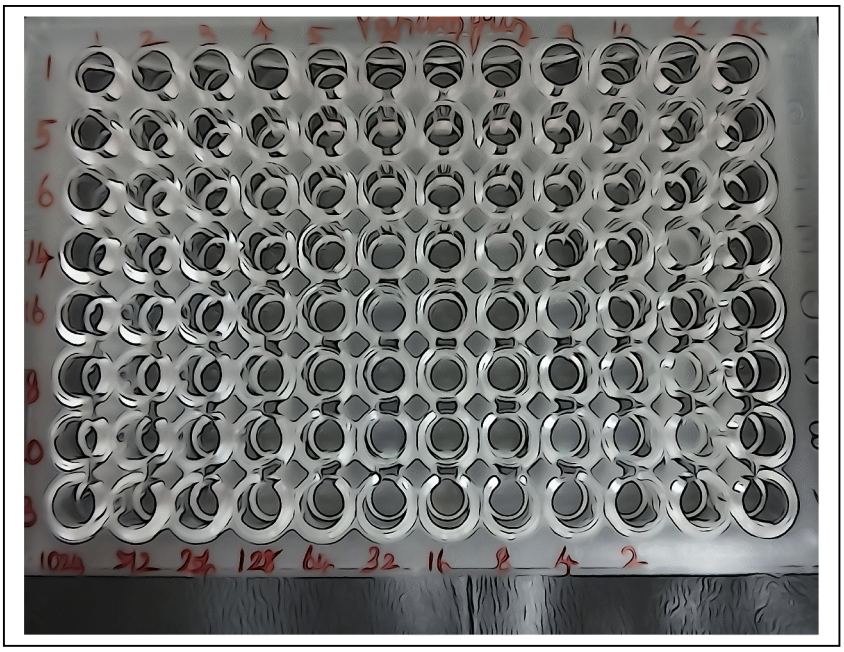


ANTIBIOTIC SUSCEPTIBILITY PATTERN OF E.faecium

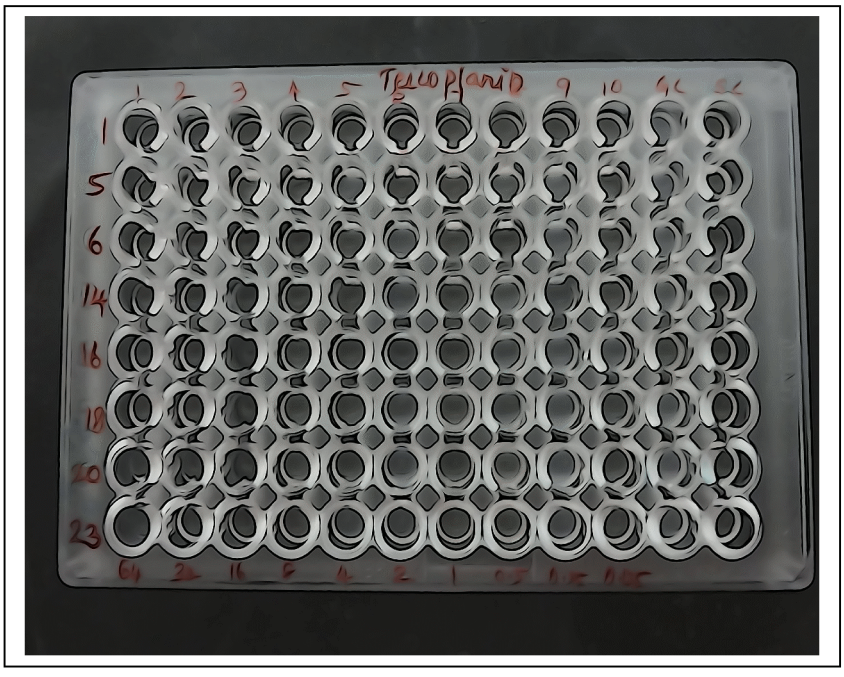
VANCOMYCIN SCREEN AGAR SHOWING POSITIVE GROWTH OF SAMPLE 23



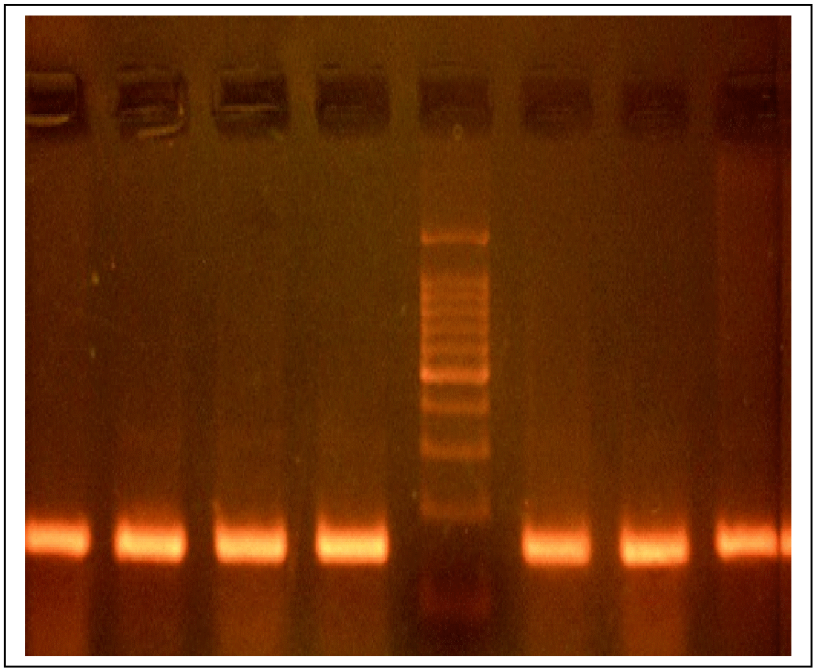
MIC TESTING OF VANCOMYCIN MICROBROTH DILUTION METHOD



MIC TESTING OF TEICOPLANIN-MICROBROTH DILUTION METHOD

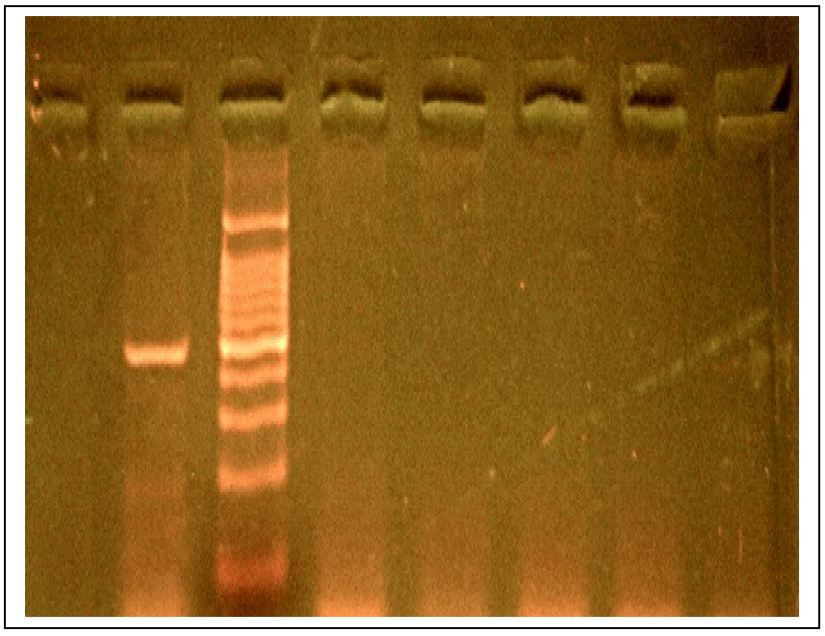


PCR- Van B gene



147 bp

PCR- Van A gene



473 bp

RESULTS

Urine, blood, pus and wound swab samples collected from 482 cases admitted at Govt. Rajaji Hospital, Madurai were included in this study. Both sexes of all age groups were included. Among 482 samples, 463 showed growth and 19 samples showed no growth. Out of 482 samples, 174 were from urine, 132 were from blood, 89 were from pus and 87 were from wound swab. Among 463 samples, 142 were enterococci, 146 were other gram positive cocci and 175 were gram negative bacilli.

Specimen wise isolation of Organisms (n=482)

SPECIMEN	ENTROCOCCI	OTHER GPC	GNB	NO GROWTH	TOTAL
URINE	58(12.0%)	28(5.8%)	81(16.8%)	7(1.4%)	174
BLOOD	32(6.6%)	46(9.54%)	49(10.16%)	5(1.0%)	132
PUS	27(5.6%)	41((8.5%)	18(3.73%)	3(0.62%)	89
WOUND SWAB	25(5.18%)	31(6.4%)	27(5.6%)	4(0.83%)	87
TOTAL	142((29.46%)	146(30.29%)	175(36.3%)	19(3.94%)	482

Specimen wise isolation of Enterococcal isolates (n=142)

SPECIMEN	NO OF ENTROCOCCAL ISOLATES	PERCENTAGE
URINE	58	40.84%
BLOOD	32	22.53%
PUS	27	19.01%
WOUND SWAB	25	17.60%

Among the 142 enterococcal isolates, 58 were isolated from urine samples, 32 from blood samples, 27 from pus samples and 25 from wound swab samples. From the above table, it is observed that Enterococci were isolated more from urine sample (40.84%) followed by blood sample (22.53%).

Age wise distribution of Enterococcal isolates (n=142)

AGE IN YEARS	NO OF PATIENTS	PERCENTAGE
< 1	3	2.11%
1 -12	23	16.19%
13 – 40	53	37.32%
41 – 60	36	25.35%
>60	27	19.01%

Analysis of age wise distribution of Enterococcal species showed predominance of enterococcal infection in middle aged group people 53 cases (37.32%).

Sex wise distribution and adult -children distribution among Enterococcal species (n= 142)

	ADULT	CHILDREN	TOTAL
MALE	64	18	82(57.7%)
FEMALE	52	8	60(42.3%)
TOTAL	116(81.7%)	26(18.3%)	142

Out of 142 enterococcal isolates, 82 (57.7%) were isolated from male and 60(42.3%) were isolated from female. 116 (81.7%) of adults and 26 (18.3%) of children were affected by Enterococcal infection.

Distribution of Enterococcal species among the specimen (n=142)

SPECIMEN	E.faecalis	E.faecium	E.raffinosus	E.sulfureus	TOTAL
URINE	34(23.94%)	19(13.38%)	4(2.82%)	1(0.70%)	58
BLOOD	18(12.68%)	12(8.45%)	2(1.4%)	0.0	32
PUS	16(11.26%)	8(5.63%)	2(1.4%)	1(0.70%)	27
WOUND SWAB	18(12.68%)	6(4.22%)	0.0	1(0.70%)	25
TOTAL	86(60.56%)	45(31.69%)	8(5.63%)	3(2.11%)	142

E. faecalis 86 (60.56%) was the predominant species followed by E. faecium, 45 (31.69%). Other enterococcal species isolated were E. raffinosus from urine, blood and pus and E. sulfureus from urine, pus and wound swab.

Screening of Enterococccal species for Bio film production by different phenotypic methods. (n=142)

BIOFILM PRODUCER /NONPRODUCER	BIO FILM PRODUCTION	TCP	TM	CRA
NO OF BIO FILM PRODUCERS	HIGH	30(21.31%)	26(18.31%)	19(13.38%)
	MODERATE	22(15.49%)	14(9.86%)	12(8.45%)
	TOTAL	52(36.62%)	40(28.17%)	31(21.83%)
NO OF NONBIO FILM PRODUCERS	WEAK/NONE	90(63.38%)	102(71.83%)	111(78.17%)

From the above table, it is observed that Tissue culture plate method detected more biofilm producer in 52 samples (36.62%) followed by Tube method and Congo red agar method.

Species and Specimen wise distribution of Biofilm producing Enterococci (n=52)

SPECIMEN	E.faecalis	E.faecium	TOTAL
URINE	21(40.38%)	4(7.7%)	25
BLOOD	8(15.38%)	—	8
PUS	7(13.46%)	2(3.8%)	9
WOUND SWAB	10(19.23%)	—	10
TOTAL	46(88.46%)	6(11.54%)	52

Out of 52 biofilm producing enterococci, 46 (88.46%) constituted E. faecalis and only 6 (11.54%) constituted E. faecium. E.raffinosus and E.sulfureus did not produce any biofilms. Biofilm producers were more isolated from urine samples followed by wound swab samples.

Comparison of different phenotypic methods

METHOD	TRUE(+VE)	FALSE(-VE)	FALSE(+VE)
TCP	50	--	2
TM	29	6	5
CRA	10	14	7

From the above table, it was found that Tissue culture plate method showed only two false positives compared to other methods.

Sensitivity and Specificity of various phenotypic methods

Method	Sensitivity	Specificity	PPV	NPV
TCP	100%	97.82%	96.15%	100%
TM	82.86%	95.32%	85.29%	94.44%
CRA	41.67%	94.07%	58.82%	88.80%

From the above table, it was observed that Tissue culture plate method had the highest sensitivity (100%) and specificity (97.82%) with PPV and NPV of 96.15% and 100% respectively.

Tube Method showed 82.86% sensitivity and 95.32% specificity with 85.29% PPV and 94.44% NPV. Congo Red Agar method had the least sensitivity (41.67%) and specificity (94.07%) with PPV and NPV of 58.82% and 88.8% respectively.

Antibiotic susceptibility pattern of Enterococcus species by Kirby Bauer disc diffusion method

ENTERO COCCUS SPECIES	TOTAL ISOLATES	AMPI		CIP		DOXY		HLS		HLG		TEICO		VANCO	
		S	R	S	R	S	R	S	R	S	R	S	R	S	R
E.faecalis	**86**	**72 (84%)**	**14 (16%)**	**21 (24%)**	**65 (76%)**	**18 (21%)**	**68 (79%)**	**57 (66%)**	**29 (34%)**	**46 (53%)**	**40 (47%)**	**78 (91%)**	**8 (9%)**	**82 (95%)**	4 (5%)
E.faecium	**45**	**12 (27%)**	**33 (73%)**	**8 (18%)**	**37 (82%)**	**17 (38%)**	**28 (62%)**	**35 (78%)**	**10 (22%)**	**16 (36%)**	**29 (64%)**	**42 (93%)**	**3 (7%)**	**30 (67%)**	15 (33%)
E.raffinosus	**8**	**5 (63%)**	**3 (37%)**	**4 (50%)**	**4 (50%)**	**5 (63%)**	**3 (37%)**	**6 (75%)**	**2 (25%)**	**6 (75%)**	**2 (25%)**	**6 (75%)**	**2 (25%)**	**8 (100%)**	0 (0%)
E.sulfureus	**3**	**1 (33%)**	**2 (67%)**	**2 (67%)**	**1 (33%)**	**0 (0%)**	**3 (100%)**	**2 (67%)**	**1 (33%)**	**3 (100%)**	**0 (0%)**	**3 (100%)**	**0 (0**	**3 (100%)**	**0 (0%)**

According to the CLSI guidelines, results were interpreted by measuring the zone of inhibition of growth around each discs. Most of the E. faecalis were sensitive to ampicillin (84%), high level streptomycin (66%), high level gentamicin (53%), teicoplanin (91%) and vancomycin (95%). They were resistant to ciprofloxacin (76%) and doxycycline (79%). Antimicrobial susceptibility pattern of E. faecium showed resistance to ampicillin (73%), ciprofloxacin (82%), doxycycline (62%) and high level gentamicin (64%) and sensitivity to high level streptomycin (78%), teicoplanin (93%) and vancomycin (67%). E. faecium showed more antimicrobial resistance than E. faecalis. Most of the E.raffinosus were sensitive to ampicillin (63%), doxycycline (63%), high level streptomycin (75%), high level gentamicin (75%), teicoplanin (75%), vancomycin (100%) and ciprofloxacin (50%). E.sulfureus were sensitive to ciprofloxacin (67%), high level streptomycin(67%),high level gentamicin(100%), teicoplanin (100%) and vancomycin(100%) and resistant to ampicillin (67%) and doxycycline(100%).5% of E. faecalis and 33% of E. faecium isolates were vancomycin resistant.

High level aminoglycoside resistance (HLAR) among E. faecalis and E. faecium isolates by disc diffusion method.

ENTEROCOCCAL SPECIES	TOTAL ISOLATES	RESISTANCE TO BOTH HLS & HLG	RESISTANCE TO HLS ONLY	RESISTANCE TO HLG ONLY	TOTAL HLAR
E.faecium	86	15	18	21	54(63%)
E.faecalis	45	10	9	18	37(82%)
TOTAL	131	25(19%)	27(21%)	39(30%)	91(69%)

The results were interpreted as per CLSI guidelines by measuring the zone of inhibition around each discs. The total high level aminoglycoside resistance (HLAR) observed was 69%. 19% isolates showed resistance to both high level streptomycin and high level gentamicin. 21% isolates showed only resistance to HLS and 30% isolates showed only resistance to HLG.

All the 142 enterococcal isolates were screened on Vancomycin screen agar for presumptive identification of vancomycin resistance. 19 isolates which showed positivity in Vancomycin screen agar, subjected to minimum inhibitory concentration (MIC) for Vancomycin and Teicoplanin as per CLSI guidelines and MIC results were interpreted to detect phenotypic classification of Vancomycin resistance.

MIC values of Vancomycin for the VRE isolates

ENTERO COCCAL Species	VRE Isolates	Vancomycin MIC Value µg/ml							TOTAL
		Intermediate 8 -16 µg/ml		Resistant > 32 µg/ml					
		8 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml	256 µg/ml	512 µg/ml	
E.faecium	15	-	1	2	4	6	1	1	15
E.faecalis	4	-	1	1	2	-	--	--	4
TOTAL	19	-	2	3	6	6	1	1	19

Out of 19 VRE isolates, 2 isolate fall within the intermediate range of 8-16 µg/ml and remaining 17 isolates fall within the resistant range of 32 – 512 µg/ml, interpreted as per CLSI guidelines.

MIC value of Teicoplanin for the VRE isolates

ENTERO COCCAL species	VRE Isolates	Teicoplanin MIC Value µg/ml						Resistant ≥32 µg/ml	TOTAL
		Susceptible					Intermediate 16 µg/ml		
		0.5 µg/ml	1 µg/ml	2 µg/ml	4 µg/ml	8 µg/ml			
E.faecium	15	7	6	0	0	--	--	2	15
E.faecalis	4	2	1	0	--	--	1	--	4
TOTAL	19	9	7	0	0	--	1	2	19

MIC - Minimum inhibitory concentration

As per CLSI guidelines, results were interpreted. Out of 19 VRE isolates the Teicoplanin MIC of 16 isolates fall within the susceptible range (0.5 – 1 µg/ml), 1 isolates fall within intermediate range (16µg/ml) and 2 isolates fall within resistant range (> 32 µg / ml).

Among 19 VRE isolates, 16 isolates showed vancomycin MIC > 16 – 512µg/ml and Teicoplanin MIC 0.5 – 1 µg/ml were identified as Van B phenotype. The remaining 3 isolates showed Vancomycin MIC > 64 µg / ml and Teicoplanin MIC >16µg/ml were identified as Van A phenotype.

Distribution of Van A and Van B genotypes in the VRE isolates by PCR assay

ENTEROCOCCAL ISOLATES	TOTAL VRE ISOLATES	Van A GENOTYPE		Van B GENOTYPE	
		PRESENT	ABSENT	PRESENT	ABSENT
E.faecium	15	2(13.33%)	13	13(86.67%)	2
E.faecalis	4	1(25%)	3	3(75%)	1
TOTAL	19	3(15.79%)	16(84.21%)	16(84.21%)	3(15.79%)

Out of 15 E. faecium VRE isolates, 2 were Van A genotype positive and 13 were Van B genotype positive. Out of 4 E. faecalis VRE isolates, 1 showed Van A genotype and 3 isolates showed Van B genotype. Van A and Van B genotype isolates showed bands at 473bp and 147bp in polymerase chain reaction (PCR) assay.

The comparison of MIC values of Vancomycin and Teicoplanin of VRE isolates with Van A and Van B genotypes.

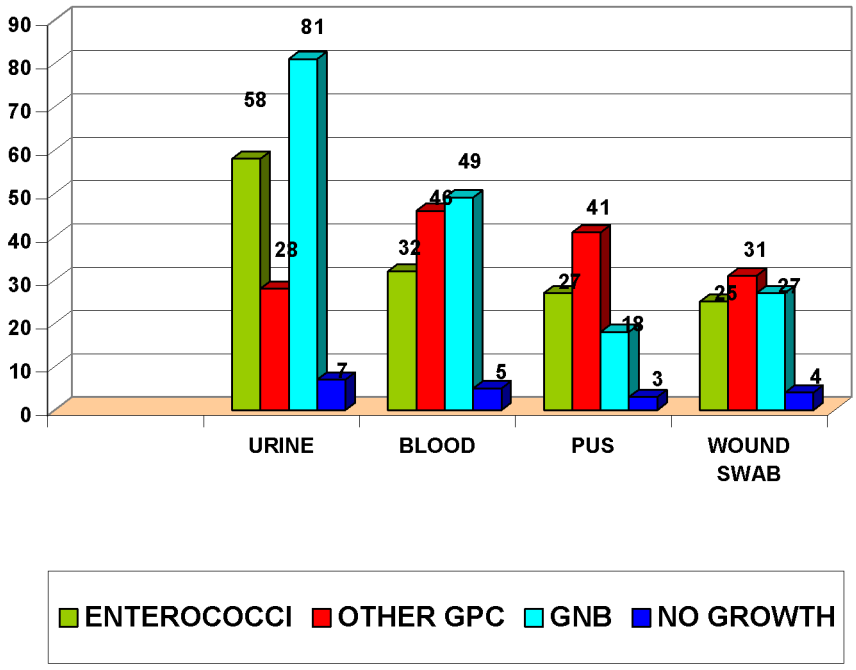
MIC VALUES	Van A GENO TYPE	Van B GENO TYPE	TOTAL
VAN MIC ≥64µg/ml TEICO MIC>16µg/ml	3	--	3
VAN MIC ≥8µg/ml TEICO MIC >0.5-1µg/ml	--	16	16
TOTAL	3(15.79%)	16(84.21%)	19

Two E. faecium isolates and one E. faecalis isolates showed high level resistance to both Vancomycin and Teicoplanin and were of Van A genotype. Thirteen E.faecium isolates and three E. faecalis isolates showed variable level resistance to Vancomycin and susceptible to Teicoplanin and were of Van B genotype.

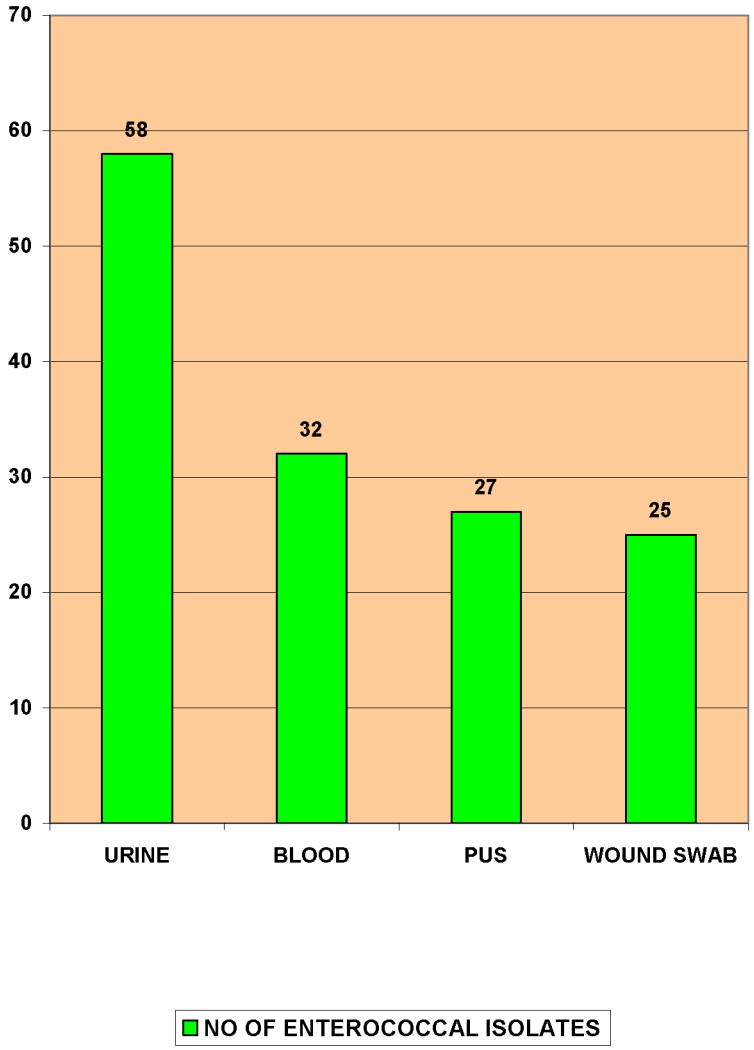
Hence, there is 100% correlation between phenotypic classification of Vancomycin resistance by Vancomycin and Teicoplanin MIC and genotypic detection of Van A and Van B resistance type by PCR assay.

All the 19 VRE isolates showed 100% sensitivity to linezolid and 85% sensitivity to chloramphenicol.

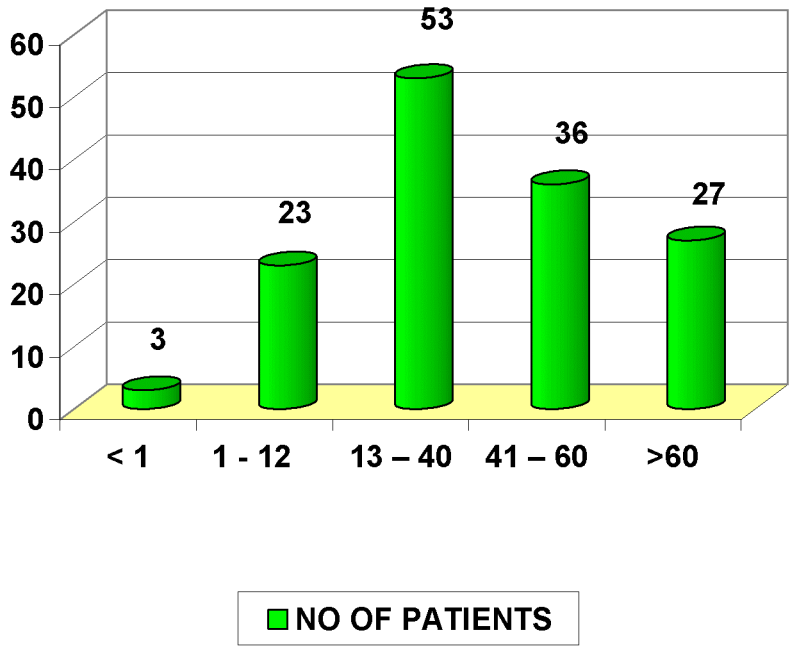
SPECIMEN WISE ISOLATION OF ORGANISMS (n=482)



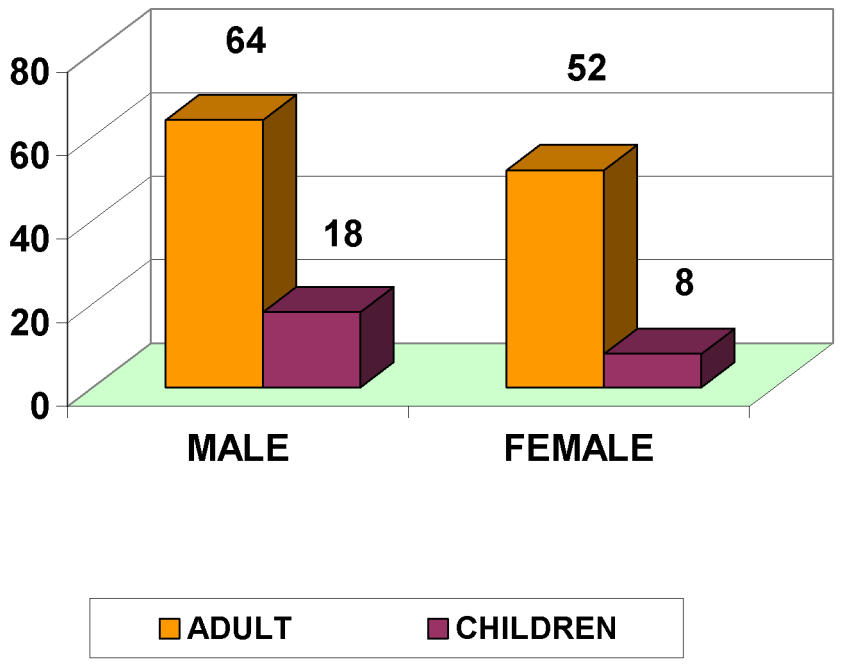
SPECIMEN WISE ISOLATION OF ENTEROCOCCAL ISOLATES



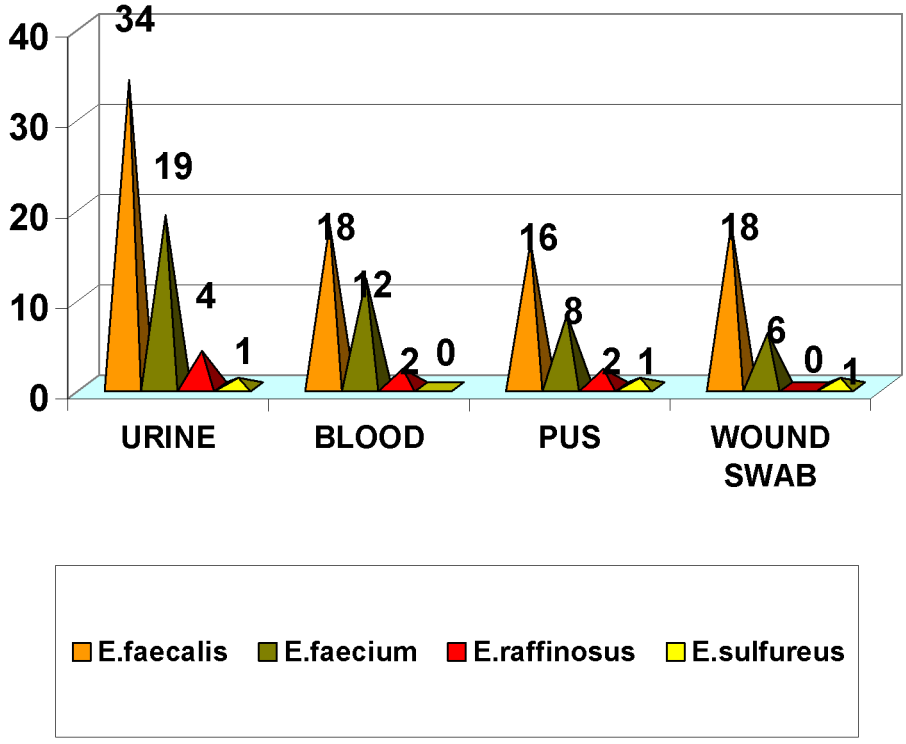
AGE WISE DISTRIBUTION OF ENTEROCOCCUS SPECIES (n=142)



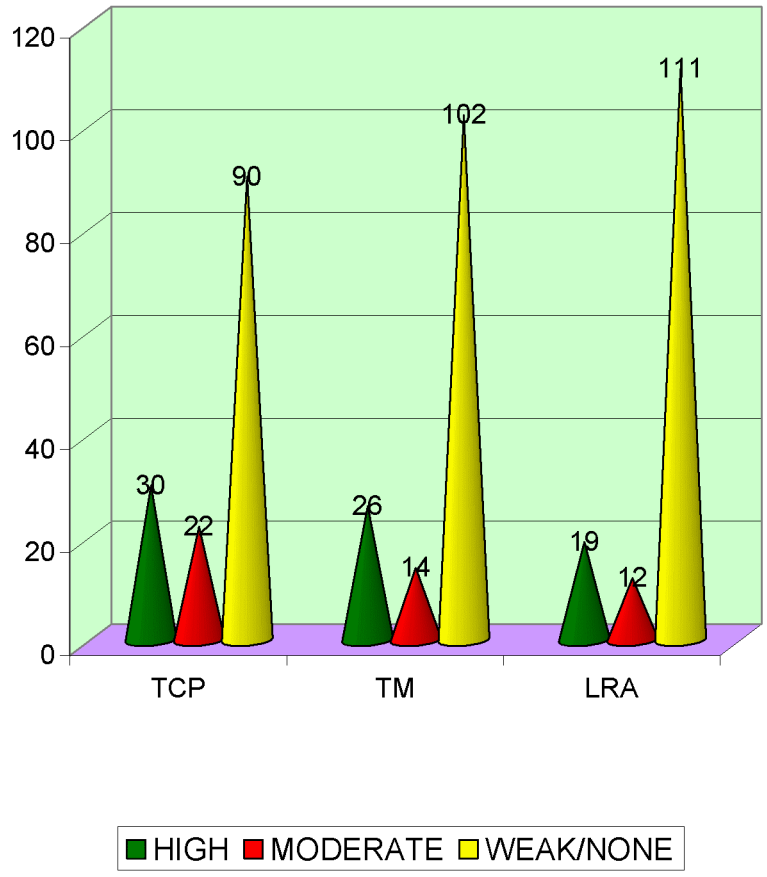
SEX WISE DISTRIBUTION AND ADULT-CHILDREN DISTRIBUTION OF ENTEROCOCCUS SPECIES (n= 142)



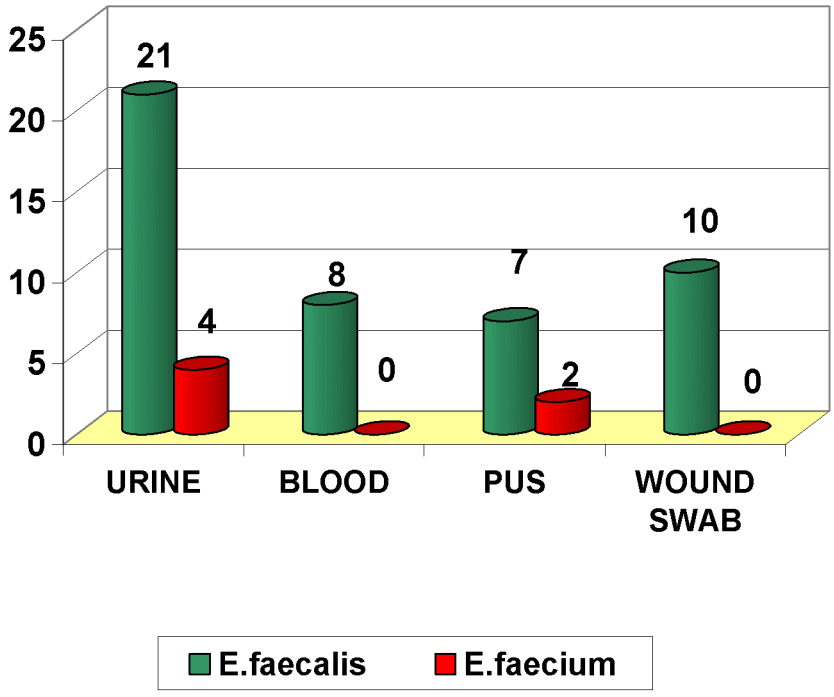
DISTRIBUTION OF ENTEROCOCCAL SPECIES
AMONG THE SPECIMENS
n=142



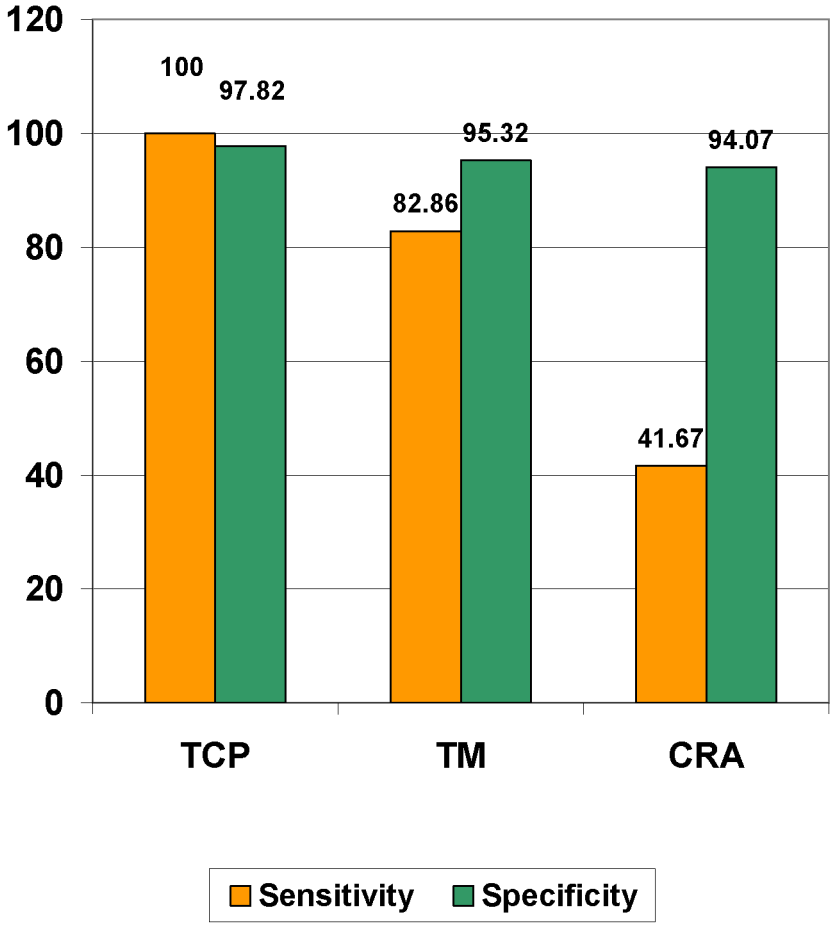
SCREENING OF ENTEROCOCCIAL SPECIES FOR BIOFILM PRODUCTION BY PHENOTYPIC METHODS (n=142)



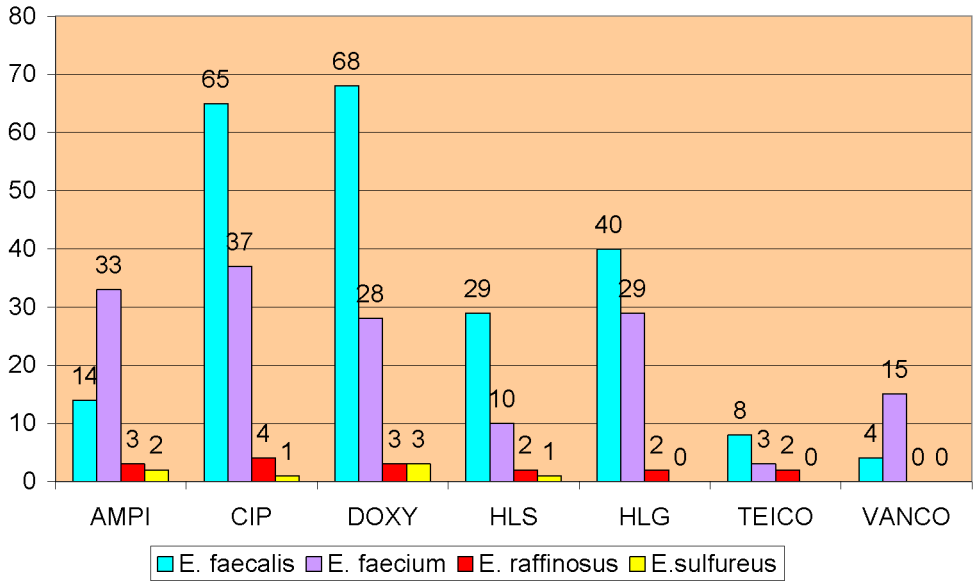
SPECIES AND SPECIMEN WISE DISTRIBUTION OF BIOFILM PRODUCING ENTEROCOCCI (n=52)



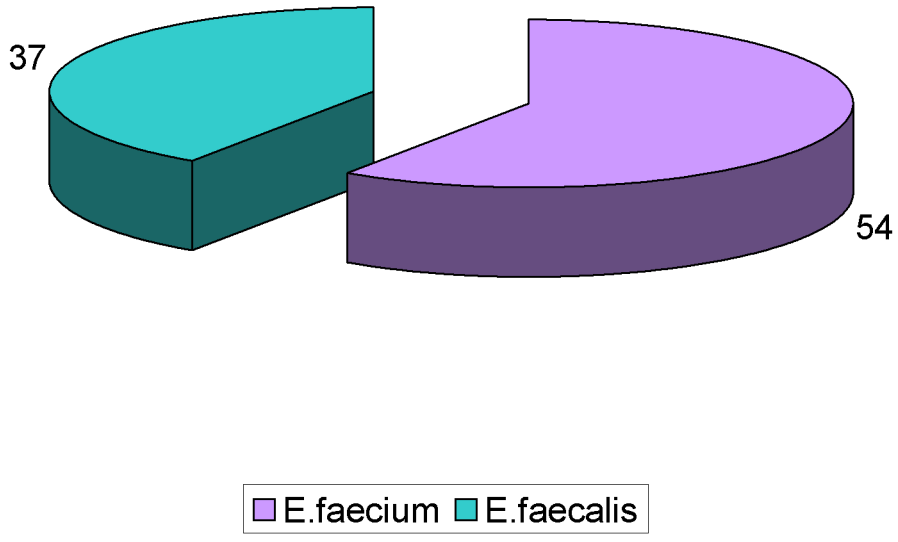
SENSITIVITY AND SPECIFICITY OF VARIOUS PHENOTYPIC METHODS



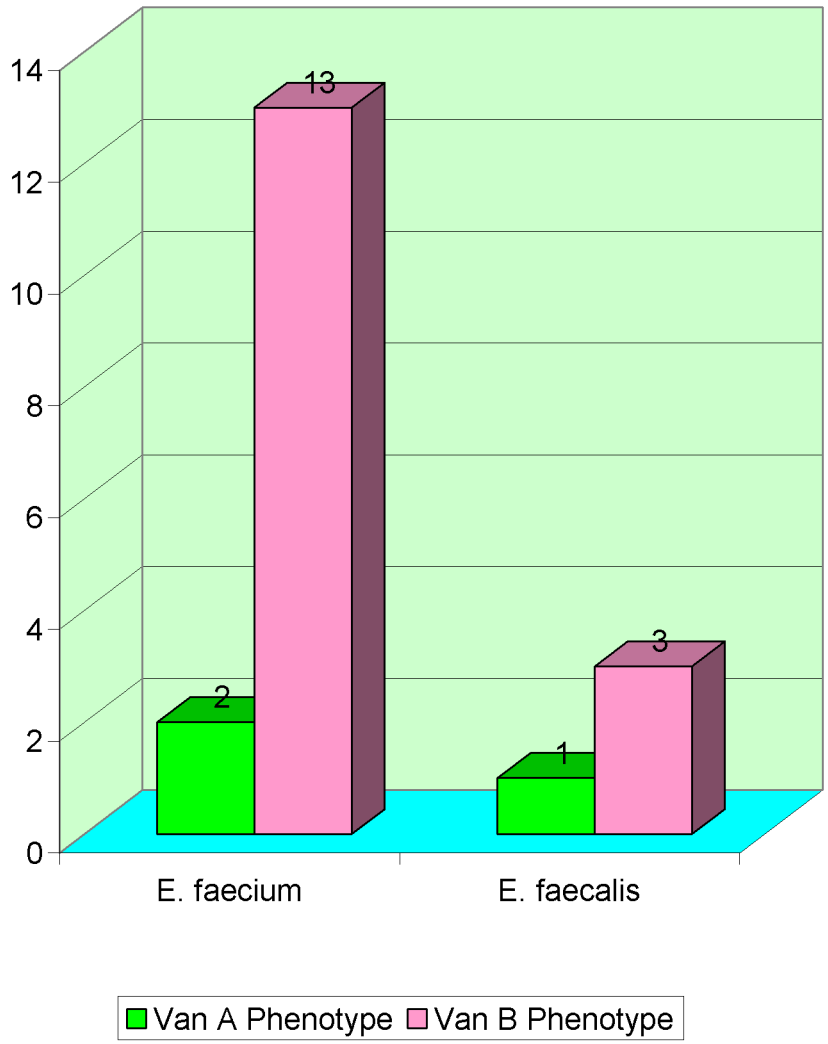
ANTI MICROBIAL RESISTANCE PATTERN OF ENTEROCOCCUS SPECIES



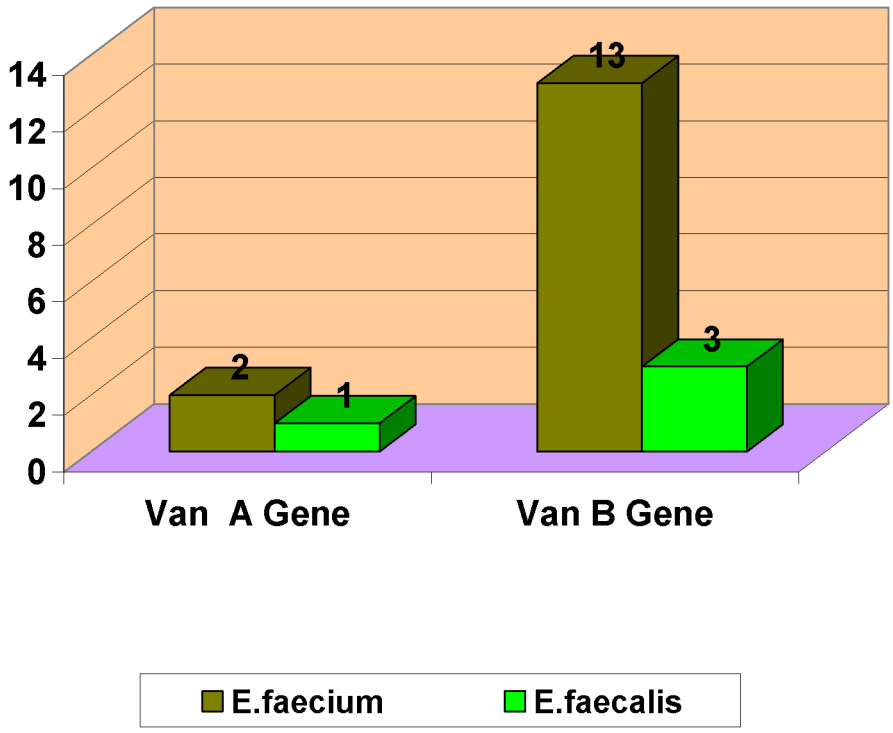
HIGH LEVEL AMINOGLYCOSIDE RESISTANCE OF
E.faecium AND E.faecalis



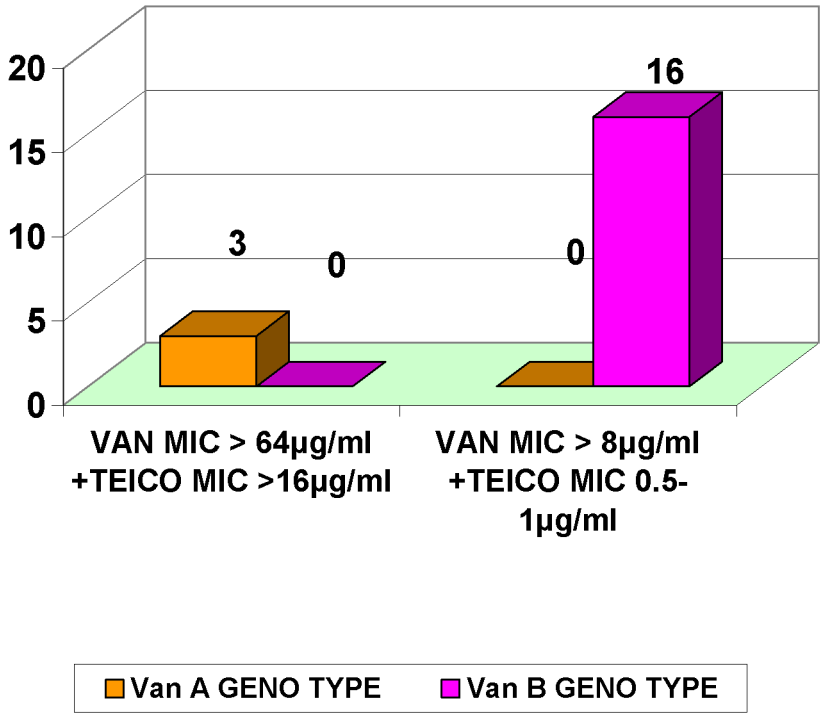
PHENOTYPIC CLASSIFICATION OF VANCOMYCIN RESISTANCE IN ENTEROCOCCI



DISTRIBUTION OF Van A AND Van B GENOTYPES IN VRE ISOLATES



COMPARISON OF MIC VALUES OF VANCOMYCIN AND TEICOPLANIN OF VRE ISOLATES WITH Van A AND Van B GENOTYPES



DISCUSSION

Enterococci have emerged as an increasingly important cause of nosocomial infections. Urinary tract infections followed by intra abdominal and pelvic infections are the commonly encountered nosocomial infections caused by these organisms. They also cause bacteremia, surgical wound infections, endocarditis, neonatal sepsis and rarely meningitis. The major reason for the prolonged survival of these organisms in hospital environment is the intrinsic resistance to several antibiotics used commonly and their ability to acquire resistance to all currently available antibiotics either by gene transfer by plasmids and transposons or by mutation. The great concern about these organisms is the emergence of Vancomycin resistance. As Vancomycin resistant enterococci (VRE) also have ampicillin resistance and high level aminoglycoside resistance, they become most difficult to treat.

More antibiotic resistance makes these pathogens excellent survivors in hospital environment and cause nosocomial infections. In this study, biofilm producing ability of Enterococci

and prevalence of Vancomycin resistance among the clinical enterococcal isolates have been assessed.

In the present study, out of 482 samples processed, 142 (29.46%) were Enterococcal species. Other organisms isolated were 146 (30.29%) other gram positive cocci and 175 (36.3%) gram negative bacilli.

Among 142 clinical Enterococcal isolates, 58 (40.84%) were from urine, 32 (22.53%) from blood, 27 (19.01%) from pus and 25 (17.60%) from wound swab samples. The results were correlated with the study by **Baragundi MC et al** who observed out of 120 enterococcal isolates, 50 were isolated from Urine sample, 35 were from blood sample, 20 were from pus sample and 15 were from wound swab samples [11]. In both studies, isolation rate of Enterococci were more from urine samples followed by blood samples.

It was analysed that more number of Enterococcal species were isolated from 16-40 years (37.32%) of age followed by 41-60 years (25.35%). Sex wise distribution of Enterococci showed that most of the enterococcal infections were associated with males 82(57.7%) than females 60(42.3%). In **Kayoko Hayakawa**

et al study, mean age of the study population affected by Enterococcal infection was in the range of 45-60 years and most of the affected people were male (53.6%).[37]

Analysis of adult-children distribution showed incidence of Enterococcal infections were more common in adults 116 (81.7%) than children 26 (18.3%).

The present study detected 86 (60.56%) E. faecalis, 45 (31.69%) E. faecium, 8 (5.63%) E. raffinosus and 3(2.11%) E.sulfureus from various clinical samples**.** The results were similar to the study by **Rahandale et al** and they documented in their study that 79 (64.23%) of E. faecalis, 40 (32.52%) of E. faecium, 1 (0.81%) of E. raffinosus and 3 (2.44%) of E. gallinarum [67].

Study conducted by **MM Salem Bekhit et al** showed 69.2%(166) of E. faecalis, 11.3%(27) of E. faecium, 2.1%(5) of E. avium, 0.8%(2) of E. hirae and 1.3%(3) of both E. casseliflavus and E. gallinarum [53].In all these studies, E. faecalis was found to be the predominant pathogen followed by E. faecium.

Bacteria in biofilms causes a more persistent infections which respond poorly to conventional antibiotic therapy. Biofilm

formation is commonly regulated by inter and intraspecies quorum sensing mechanisms.

The present study showed 52(36.62%) out of 142 Enterococcal isolates were biofilm producers.Out of 52 biofilm producers, 30 (21.31%) were high biofilm producer and 22 (15.49%) were moderate biofilm producer. In **Giridhara upadhyaya PM et al** study they documented that out of 65 biofilm producer, 23 (11.5%) were high biofilm producer and 42(21%) were moderate biofilm producer.[63]

In the present study, out of 52 biofilm formers, 46 (88.46%) were E. faecalis and 6 (11.54%) were E. faecium. This result was in discordance with the study of **Jonathan A.T. Sandoe et al** and in their study they documented 100% E. faecalis and 42% E. faecium formed biofilms.[32]

In this study, the percentage of biofilm production detected by Tissue culture plate method (36.62%) was high followed by tube method (28.17%) and Congo red agar method (21.83%). This finding correlated with **Mathur et al** showed that the number of biofilm producers identified by TCP method was high (53.9%) and followed by TM (11.8%) and CRA method.[49] In another

study conducted by **Manpreet Kour et al**, noted that 80.8% biofilm producer detected by TCP method, 43.9% by TM and 27.7% by CRA method.[48] **Knobloch et al, HiHinahalli et al, Ira et al** found that TCP method to be more accurate and sensitive for biofilm detection as compared to TM and CRA method. The sensitivity and specificity of TCP method, TM and CRA method were 100%, 82.86%, 41.67% and 97.82%, 95.32% and 94.07% respectively. In **Soni et al** study, the sensitivity and specificity of TCP method, TM, CRA method were 94%, 77%, 38% and 83%, 81% and 44% respectively.[48]

In the present study, antimicrobial susceptibility pattern of E. faecium showed 73% ampicillin resistance. **Agarwal J et al** have reported significantly higher resistance to ampicillin among E. faecium isolates which was similar to our study.[3] In their study they documented multidrug resistant enterococci which was a similar finding in our study.

In the present study, the total high level aminoglycoside resistance (HLAR) observed was 69%. This finding is similar to the report by **Kapoor et al** and in their study they reported 66% of total HLAR.[35]

63% of E. faecium and 82% of E. faecalis showed total HLAR in our study. In **Mohanty S et al** study, 81% of E. faecium and 72% of E. faecalis isolates exhibited HLAR.[55]

In this study, 21% of resistance to high level streptomycin only and 30% of resistance to high level gentamicin only were reported.

Among 142 enterococcal isolates,19 were identified as vancomycin resistant on vancomycin screen agar. These isolates were subjected to detection of minimum inhibitory concentration for Vancomycin and Teicoplanin as a confirmatory test.

In broth microdilution technique, 2 isolates showed Vancomycin MIC in intermediate range (16 µg / ml) and remaining 17 isolates showed Vancomycin MIC in resistant range (> 32 – 512 µg / ml). For Teicoplanin, 16 isolates showed susceptible range of 0.5-1 µg/ml, 1 isolate showed intermediate range of 16 µg /ml and 2 isolates showed resistant range of greater than 32 µg/ml.

About 13 E. faecium isolates and 3 E. faecalis isolates showed variable range of resistance to Vancomycin (MIC 16-512µg/ml) and susceptible to Teicoplanin (0.5 – 1 µg/ml) and are

of Van B phenotype. 2 isolates of E. faecium and 1 isolate of E. faecalis showed high level resistance to both Vancomycin (MIC > 64 µg/ml) and Teicoplanin (MIC > 16 µg/ml) and are of Van A phenotype. In this study, VanB phenotype was more prevalent than VanA phenotype. This result was similar to the study conducted by **Taneja N et al, Kapoor et al and Karmarker MG et al** and all these studies showed higher isolation of VanB phenotype than VanA phenotype [83,35,36].

All the 19 VRE isolates were subjected to polymerase chain reaction (PCR) which showed 13 E. faecium and 3 E. faecalis were of Van B genotype and 2 E. faecium and 1 E. faecalis were of Van A genotype. The present study shown higher isolation rate of VanB genotype 84.2% (16/19) than VanA genotype 15.7% (3/19). This result was similar to the study by **Nelson et al** who documented that 97% isolates were Van B positive genotype and the remaining isolates were Van A genotype [59].

The prevalence of VRE in this study was 13.3% which was higher than the results shown by **Baragundi et al, Taneja N et al, Kapoor et al** ,they reported 7.5%, 5.5% and 8% of VRE

respectively [11,83,35] and was lower than the study of **Karmarker MG et al** ,they reported 23% VRE [37].

All the 19 VRE isolates showed 100% sensitivity to linezolid. This result was similar to **MM Salem.Bekhit et al** study and they reported 100% sensitivity to linezolid by all VRE isolates.

SUMMARY

- A total of 482 samples were collected to study biofilm production and Vancomycin resistance in Enterococcal isolates.
- A total of about 142 Enterococcal isolates were isolated from clinical specimens. Majority of the isolates were from urine 40.84% followed by blood 22.53%, pus 19.01% and wound swab 17.60%.
- Adults 116 (81.7%) were affected more than the children 26 (18.3%) by Enterococcal infection. Higher isolation rate of about 57.7% (82/142) was observed in male patients when compared to female patients 42.3% (60/142).
- In the present study, E. faecalis was the predominant species with an isolation rate of about 60.56% followed by E. faecium 31.69%. Other species isolated were E. raffinosus (5.63%) and E. sulfureus (2.11%). E. faecalis had the highest isolation in urine samples.
- Phenotypic detection of biofilm production among Enterococcal isolates were high in Tissue culture plate method when compared to Tube method and Congo red agar method.
- Predominant biofilm producer was E. faecalis 88.46% (46/52) followed by E. faecium 11.54% (6/52).

- Among the phenotypic method, Tissue culture plate method had the highest sensitivity and specificity as compared with other phenotypic methods.
- In antibiotic susceptibility testing, E. faecium showed 73% resistance to penicillin, 82% resistance to ciprofloxacin, 62% resistance to doxycycline, and 64% resistance to high level gentamicin. 76% resistance to ciprofloxacin and 79% resistance to doxycycline showed by E. faecalis isolates.
- The high level aminoglycoside resistance (HLAR) observed was 69%. Resistance to both high level streptomycin and high level gentamicin observed in 19% (25/91) isolates. 21% of resistance to high level streptomycin only and 30% of resistance to high level gentamicin only was observed.
- The high level aminoglycoside resistance was higher in E. faecalis isolates 82% (37/45) than E. faecium isolates 63% (54/86).
- About 19 isolates (13.3%) were presumptively identified as Vancomycin resistant by Vancomycin screen agar.
- Minimum inhibitory concentration of Vancomycin by microbroth dilution technique of 19 isolates showed 2 isolates in intermediate

range (MIC 8-16 µg/ml) and 17 isolates in resistant range (MIC > 32-512 µg/ml)

- Minimum inhibitory concentration of Teicoplanin by microbroth dilution method of 19 isolates showed 16 isolates in susceptible range (MIC 0.5 – 1 µg/ml) and 1 isolate in intermediate range (MIC 16µg/ml) and 2 isolates in resistant range(MIC>32µg/ml)
- Among 19 VRE isolates, 16 isolates showed vancomycin MIC > 16 – 512µg/ml and Teicoplanin MIC 0.5 – 1 µg/ml were identified as Van B phenotype.
- The remaining 3 isolates showed Vancomycin MIC > 64 µg / ml and Teicoplanin MIC >16µg/ml were identified as Van A phenotype.
- Total VRE isolates as per Vancomycin MIC value were 19 and the prevalence rate was 13.3% (19/142).
- All the 19 VRE isolates were subjected to Polymerase Chain Reaction for the detection of vancomycin resistance genes.
- In PCR assay, a total of 16 isolates including 13 E. faecium and 3 E. faecalis were found to be of Van B genotype and the remaining 3 isolates including 2 E. faecium and 1 E. faecalis were found to be of Van A genotype.

- The present study showed 100% correlation between phenotypic classification of Vancomycin resistance by detecting Vancomycin and Teicoplanin MIC and genotypic detection of Van A and Van B genes of VRE by PCR assay.
- All the 19 VRE isolates were sensitive to linezolid(100%) and chloramphenicol(85%).

CONCLUSION

- Enterococci are one of the common organisms causing nosocomial infections at Govt. Rajaji Hospital, Madurai Medical College, Madurai.
- E.faecalis is the most commonly isolated species followed by E.faecium.
- On evaluation of bioform forming ability of Enterococci by three phenotypic methods,Tissue culture plate method showed high sensitivity and specificity of 100% and 97.82% respectively.
- Since Tissue culture plate method was proved to be a simple and cost effective method,it can be recommended for the early diagnosis of biofilm formation.
- E.faecium isolates showed more ampicillin(73%),high level aminoglycoside(63%) and vancomycin(33%) resistance than E.faecalis.
- In this study,the prevalence of Vancomycin resistance in Enterococcal species is 13.3% as per vancomycin MIC by Microbroth dilution technique.

- The phenotypic detection of Vancomycin resistance by MIC of Vancomycin and Teicoplanin correlates with the genotypic method of detection of Vancomycin resistance genes.
- In molecular method limited settings, phenotypic detection of Vancomycin resistance by Vancomycin and Teicoplanin MIC can be recommended.
- The emergence of Vancomycin resistant enterococci have posed serious threats to the community because they exhibit multiple drug resistance, thus limiting the therapeutic options for the clinicians.
- Judicious use of Vancomycin, rapid isolation of patients suspected to have VRE infections and regular testing of all Enterococcal isolates for vancomycin resistance is recommended for the prevention of nosocomial transmission of VRE.
- Appropriate surveillance, stringent infection control measures and continuous monitoring is very important to control the spread and cross contamination of Vancomycin Resistant Enterococci.

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ANNEXURE – 1
PREPARATION OF GRAM STAIN

GRAM STAIN REAGENTS

1. Methyl violet – Primary stain

   Methyl violet - 10g

   95% Ethyl alcohol- 100ml

   Distilled water -1 L

2. Gram's Iodine - Mordant

   Iodine - 10g

   Potassium Iodide - 20g

   Distilled water -1 L

3. Acetone - Decolouriser

4. Dilute carbol fuchsin – Counter stain

   Basic fuchsin - 0.3 g

   95% Ethyl alcohol - 10 ml

   Phenol crystals, melted- 5 ml

   Distilled water -95 ml

Basic fuchsin was dissolved in alcohol. 5% phenol solution was added and allowed to stand overnight. This solution was filtered through coarse filter paper.

ANNEXURE – 2

PREPARATION OF MEDIA

Bile Esculin Agar plate :

Ingredients :

Peptone	-	5 gm
Beef extract	-	3 gm
Oxgall (bile)	-	40 gm
Esculin	-	1 gm
Ferric citrate	-	0.5 gm
Agar	-	15 gm
Distilled water	-	1 L
pH	-	7.0

All the contents were dissolved completely by heating and sterilized at 121°C for 10 minutes in autoclave. Then the medium was poured into slants or petri dish plates.

Congo Red Agar (CRA) plate :

To prepare 1 L of CRA médium, need

1. Brain heart infusion broth 37g / L
2. Sucrose 50g/L
3. Agar No.1 10g / L
4. Congo red indicator 8g / L

First Congo red stain was prepared as a concentrated aqueous solution and autoclaved at 121° C for 15 minutes separately

from the other medium constituents. Then it was added to the autoclaved brain heart infusion agar with sucrose at 55°C.

Vancomycin screen Agar plate

Ingredients :

1. Agar - 15 gm
2. Brain heart infusion broth – 1L
3. Vancomycin – 6 mg / L

The agar was dissolved in brain heart infusion broth completely by boiling. Then the medium was autoclaved at 121°C for 15 minutes and cooled to about 50°C. Vancomycin 6µg / ml was added, mixed well and poured into petri dish plates.

ANNEXURE – 3

DATA COLLECTION PROFORMA

1. CASE No :

2. NAME :

3. AGE/SEX :

4. ADDRESS :

5. IP/OP No :

6. WARD/UNIT :

7. DATE OF ADMISSION :

8. CLINICAL DIAGNOSIS :

9. BRIEF CLINICAL HISTORY :

10. PAST HISTORY :

11. PERSONAL HISTORY :

12. TREATMENT HISTORY :

13. SAMPLE COLLECTED :

14. DATE OF SAMPLE COLLECTION :

MICROBIOLOGICAL INVESTIGATIONS

1. DIRECT SMEAR STUDY (GRAM'S STAIN) – PUS CELLS, EPITHELIAL CELLS, BACTERIAL MORPHOLOGY
2. GROWTH ON CULTURE MEDIA
   - I. NUTRIENT AGAR PLATE.
   - II. MACKONKEY AGAR PLATE.
   - III. BLOOD AGAR PLATE.
   - IV. BILE ESCULIN AGAR PLATE

3. GRAM'S STAIN FROM CULTURE :
4. CATALASE TEST :
5. HEAT TOLERANCE TEST :
6. SALT TOLERANCE TEST :
7. MANNITOL MOTILITY TEST :
8. ARGININE DIHYDROLYSIS TEST :
9. CARBOHYDRATE (1%) FERMENTATION TEST :
10. DETECTION OF BIOFILM FORMATION.

BIOFILM PRODUCTION	TISSUE CULTURE PLATE	TUBE METHOD	CONGO RED AGAR
HIGH			
MODERATE			
WEAK/NONE			

11. ANTIMICROBIAL SUSCEPTIBILITY TESTING

AMPI		CIP		DOXY		HLS		HLG		TEICO		VAN	
S	R	S	R	S	R	S	R	S	R	S	R	S	R

12. DETECTION OF VANCOMYCIN RESISTANCE

VANCOMYCIN SCREEN AGAR	VANCO MIC	TEICO MIC	VAN PHENOTYPE	VAN GENOTYPE (Van A, Van B)

KEY TO MASTER CHART

U	-	URINE SAMPLE
B	-	BLOOD SAMPLE
P	-	PUS SAMPLE
WS	-	WOUND SWAB SAMPLE
UTI	-	URINARY TRACT INFECTIONS
PUO	-	PYREXIA OF UNKNOWN ORIGIN
CKD	-	CHRONIC KIDNEY DISEASE
WI	-	WOUND INFECTION
TCP	-	TISSUE CULTURE PLATE METHOD
TM	-	TUBE METHOD
CRA	-	CONGO RED AGAR METHOD
HLS	-	HIGH LEVEL STREPTOMYCIN
HLG	-	HIGH LEVEL GENTAMICIN
VSA	-	VANCOMYCIN SCREEN AGAR
MIC	-	MINIMUM INHIBITORY CONCENTRATION

						BIOFILM PRODUCTION			ANTIBIOGRAM											
S.NO:	LAB ID NO:	AGE	SEX	SPECIMEN/SPECIES	CLINICAL DIAGNOSIS	TCP	TM	CRA	AMPI	CIP	DOXY	HLS	HLG	TEICO	VAN	VSA	VAN MIC	TEICO MIC	VAN (PHENOTYPE)	VAN (PCR)
1	3970	30	M	E.faecalis-U	UTI	+	+	+	S	R	R	R	S	S	S					
2	1592	21	F	E.faecium-P	LEG ULCER				R	S	S	R	S	S	S					
3	3339	8 MON	Fch	E.faecalis-B	PUO	+	+	+	R	R	S	S	S	S	S					
4	4012	18	M	E.faecalis-U	CKD	+	+	+	S	S	R	S	R	R	S					
5	1603	42	M	E.faecalis-P	INJURY	+	+	+	S	R	R	S	S	S	S					
6	1634	54	F	E.faecalis-WS	DIABETIC ULCER	+	+	+	S	R	R	R	S	S	S					
7	4108	6	Mch	E.faecium-U	UTI		+	+	R	R	S	R	R	S	R	+	16	0.5	Van B	Van B
8	1643	29	F	E.faecalis-WS	THIGH ULCER				S	S	R	S	R	S	S					
9	3372	7	Fch	E.faecalis-B	PUO				S	R	R	S	R	S	S					
10	4302	51	M	E.faecalis-U	UTI	+	+		R	R	S	S	S	S	R	+	32	1	Van B	Van B
11	3398	22	M	E.faecium-B	PUO		+	+	R	R	R	R	R	S	S					
12	4318	33	F	E.faecalis-U	UTI				S	R	R	S	R	S	S					
13	3409	63	M	E.faecalis-B	PUO	+	+		S	S	R	R	S	S	S					
14	3421	11	Fch	E.faecalis-B	ENTERIC FEVER				S	R	R	S	S	R	R	+	64	16	Van A	Van A
15	1704	70	F	E.faecalis-P	WOUND INFECTION	+	+	+	S	R	R	S	S	S	S					
16	1726	44	F	E.faecium-WS	POST OP WI				R	R	S	R	S	S	S					
17	4354	18	F	E.faecalis-U	UTI	+	+	+	R	S	S	S	R	S	S					

18	1765	38	M	E.faecalis-P	ABSCESS-THIGH				S	R	R	S	R	S	S					
19	4390	46	M	E.raffinosus-U	UTI				R	S	S	R	S	S	S					
20	1801	65	F	E.faecalis-WS	WOUND INFECTION	+	+	+	S	R	R	R	S	S	S					
21	4441	60	F	E.faecalis-U	UTI				S	R	R	S	R	S	S					
22	4469	55	M	E.faecium-U	UTI	+	+	+	R	R	R	R	R	S	S					
23	4482	16	M	E.faecalis-U	UTI	+			S	R	R	R	S	S	S					
24	4507	62	M	E.faecium-U	PUO	+	+		R	R	S	R	S	S	S					
25	1857	19	F	E.faecalis-P	DM-ULCER				S	S	R	S	R	S	S					
26	4568	45	F	E.faecalis-U	CKD	+	+	+	R	R	R	S	S	S	S					
27	3488	10 MON	Mch	E.faecalis-B	FFE				S	R	R	S	S	S	S					
28	1902	53	M	E.faecalis-P	ABSCESS-LT LEG				S	R	S	S	R	S	R	+	16	0.5	Van B	Van B
29	3503	69	F	E.faecium-B	PUO				R	R	R	R	R	S	R	+	128	1	Van B	Van B
30	4605	25	M	E.faecalis-U	CYSTITIS	+	+	+	S	R	R	R	R	S	S					
31	1989	31	F	E.faecium-P	INJURY-RA				R	R	S	R	R	S	S					
32	3575	50	F	E.faecium-B	FFE		+	+	R	S	S	R	S	S	S					
33	4684	7	Fch	E.faecalis-U	PUO				S	R	R	S	S	R	S					
34	3611	72	M	E.raffinosus-B	FFE				R	R	S	S	R	S	S					
35	3675	12	Mch	E.faecalis-B	PUO	+	+		R	S	S	S	R	S	S					
36	4722	19	M	E.sulfureus-U	UTI				R	S	R	S	S	S	S					
37	2008	46	M	E.faecalis-WS	POST OP WI	+	+	+	S	R	R	S	S	S	S					
38	3690	8	Mch	E.faecalis-B	ENTERIC FEVER				S	R	R	S	R	S	S					
39	4782	24	F	E.faecalis-U	PID	+	+		S	R	R	R	R	S	S					
40	2024	71	F	E.faecalis-WS	WOUND INFECTION				S	R	R	S	R	S	R	+	64	0.5	Van B	Van B
41	2046	6	Fch	E.faecalis-P	ABSCESS	+	+	+	S	S	R	S	S	S	S					
42	2111	20	M	E.raffinosus-P	WOUND INFECTION				S	S	S	S	S	S	S					
43	2243	62	F	E.faecalis-WS	POST OP WI	+			S	R	S	S	S	S	S					
44	2279	75	M	E.faecalis-P	DIABETIC ULCER				S	R	R	R	R	S	S					

45	4814	31	F	E.faecalis-U	UTI	+			R	R	R	R	S	S	S					
46	3732	9 MON	Fch	E.faecalis-B	SEPTICEMIA				R	S	S	S	R	S	S					
47	4856	15	M	E.faecium-U	UTI				R	R	S	R	R	S	S					
48	4912	13	F	E.faecalis-U	PUO	+	+	+	S	R	R	R	R	S	S					
49	5045	47	M	E.faecium-U	UTI	+	+	+	R	R	R	R	S	S	R	+	256	1	Van B	Van B
50	2318	24	F	E.faecium-P	ULCER				R	S	R	R	R	S	S					
51	2434	51/5	Mch	E.faecalis-P	WOUND INFECTION	+			S	R	R	S	R	R	S					
52	5132	52	F	E.raffinosus-U	PUO				S	R	R	S	S	S	S					
53	5189	33	M	E.faecalis-U	CKD		+	+	R	R	S	R	S	S	S					
54	2566	63	M	E.faecium-WS	SURGICAL SITE INFECTION				R	R	R	R	R	S	S					
55	3817	52	M	E.faecium-B	SEPSIS				R	R	R	R	R	S	S					
56	2610	28	M	E.faecalis-WS	ULCER	+			S	S	R	S	S	S	S					
57	3972	20	F	E.faecium-B	PUO				R	S	R	R	S	R	R	+	64	32	Van A	Van A
58	4015	5	Mch	E.faecalis-B	PUO	+	+		S	R	R	S	S	S	S					
59	2721	59	F	E.sulfureus-P	ABSCESS				R	R	R	S	S	S	S					
60	5416	49	M	E.faecalis-U	UTI	+	+	+	R	S	R	S	R	S	S					
61	2914	17	M	E.faecalis-P	ULCER				S	R	R	R	S	S	S					
62	5603	67	F	E.faecalis-U	UTI	+			S	R	S	S	R	S	S					
63	3001	74	M	E.faecalis-WS	WOUND INFECTION				S	R	R	S	S	S	S					
64	5712	41	F	E.faecium-U	UTI				R	R	S	R	S	S	S					
65	5841	18	F	E.faecium-U	UTI				R	R	S	R	R	S	S					
66	5985	8	Fch	E.faecalis-U	PUO	+	+		R	R	R	R	R	S	S					
67	3074	51	F	E.faecalis-P	WOUND INFECTION				S	S	S	S	S	S	S					
68	6092	26	M	E.faecalis-U	UTI				S	R	R	S	S	S	S					
69	6149	57	M	E.faecium-U	CKD				R	S	R	R	S	S	R	+	128	0.5	VanB	Van B
70	4235	7	Mch	E.faecalis-B	SEPSIS	+	+		R	R	R	R	S	S	S					
71	6212	25	F	E.faecium-U	PID				R	R	R	R	R	S	S					

72	6336	45	F	E.raffinosus-U	PUO				S	S	S	S	S	S	S						
73	3118	72	F	E.faecalis-WS	POST OP WI	+			S	R	R	S	R	S	S						
74	3190	18	M	E.sulfureus-WS	WOUND-LL		+	+	S	S	R	R	S	S	S						
75	4444	49	M	E.faecium-B	FFE				R	R	S	R	R	S	S						
76	3263	28	F	E.faecalis-WS	ULCER				R	S	S	R	R	R	S						
77	6474	72	M	E.faeium-U	UTI	+	+	+	R	R	R	R	S	S	R	+	32	1	Van B	VanB	
78	6510	39	M	E.faecalis-U	UTI	+			S	R	R	S	S	S	S						
79	6596	3	Mch	E.faecalis-U	SEPTICEMIA				S	R	R	S	S	S	S						
80	4613	50	M	E.faecalis-B	ENTERIC FEVER				S	R	R	S	R	S	S						
81	6640	39	F	E.faecium-U	PID				R	R	S	R	S	S	R	+	64	0.5	VanB	Van B	
82	6712	3	Fch	E.faecalis-U	FFE				S	S	R	R	S	S	S						
83	3352	26	M	E.faecalis-WS	WOUND INFECTION	+	+		S	R	R	S	R	S	S						
84	3411	69	M	E.faecalis-P	CVA WITH ULCER	+	+	+	S	R	R	S	S	S	S						
85	3619	43	F	E.faecium-P	CELLULITIS				R	R	R	R	R	S	S						
86	5114	27	F	E.faecium-B	FFE				R	R	S	R	R	S	S						
87	6875	66	M	E.faecalis-U	UTI				S	R	R	R	R	S	S						
88	6891	8	Fch	E.faecalis-U	PUO	+			S	S	S	S	S	S	S						
89	3706	44	F	E.faecalis-P	ULCER	+	+		S	R	R	S	S	S	S						
90	5484	65	F	E.faecium-B	PUO				S	R	R	R	S	R	R	+	64	32	Van A	Van A	
91	3784	34	M	E.faecalis-WS	WOUND INFECTION	+	+	+	S	R	R	S	S	S	S						
92	6959	30	F	E.faecium-U	UTI				S	R	R	R	R	S	S						
93	5808	14	M	E.faecalis-B	SEPTICEMIA	+			S	S	S	S	R	R	S						
94	6992	43	M	E.faecium-U	PUO				S	R	R	R	R	S	R	+	128	0.5	VanB	Van B	
95	3819	71	M	E.faecalis-WS	POST OP WI			+	R	R	R	R	R	S	S						
96	7013	25	M	E.faecalis-U	UTI				S	R	R	S	S	S	S						
97	3856	18	F	E.faecium-P	ABSCESS	+	+	+	S	S	S	R	S	S	R	+	128	1	Van B	Van B	
98	3872	50	F	E.faecium-WS	WOUND INFECTION				S	R	R	R	R	S	S						
99	7046	63	F	E.faecium-U	UTI				S	R	S	R	R	R	S						

100	6111	11	Mch	E.faecalis-B	SEPTICEMIA			+	S	S	S	R	S	S	S					
101	3892	24	F	E.faecium-WS	WOUND INFECTION				R	R	R	R	R	S	R	+	64	0.5	VanB	Van B
102	6179	19	M	E.raffinosus-B	PUO				S	R	R	S	S	R	S					
103	3900	57	M	E.faecalis-P	CHRONIC OM				S	R	R	S	R	S	S					
104	6202	10	Fch	E.faecalis-B	FFE				S	R	R	R	S	S	S					
105	7098	61	F	E.faecalis-U	UTI	+			S	R	R	S	R	S	S					
106	114	29	M	E.faecalis-U	UTI				S	S	R	R	S	S	S					
107	74	19	F	E.faecium-P	ULCER				R	R	S	R	S	S	R	+	128	0.5	VanB	Van B
108	415	42	F	E.faecalis-U	CKD	+	+		R	R	S	S	R	S	S					
109	102	30	M	E.faecium-P	ABSCESS	+			S	R	R	R	R	S	S					
110	123	56	M	E.faecium-B	FFE				S	R	R	R	R	S	S					
111	280	13	M	E.faecalis-B	PUO	+	+	+	S	R	R	R	S	R	S					
112	214	29	M	E.raffinosus-P	ABSCESS				S	S	S	S	R	S	S					
113	296	27	F	E.faecalis-P	WOUND INFECTION	+			S	R	R	S	R	S	S					
114	574	21	M	E.faecium-U	UTI				R	S	R	R	S	S	S					
115	624	58	F	E.faecium-U	UTI				S	R	R	R	R	S	S					
116	688	6	Fch	E.faecalis-U	PUO	+	+	+	S	R	R	R	S	S	S					
117	442	10	Mch	E.faecalis-B	SEPTICEMIA				S	S	R	S	S	S	S					
118	410	47	M	E.faecium-WS	CELLULITIS				R	R	S	R	R	S	R	+	512	1	VanB	VanB
119	491	19	M	E.faecalis-WS	ULCER	+			S	R	S	S	R	S	S					
120	516	33	F	E.faecalis-WS	WOUND INFECTION				S	R	R	S	R	S	S					
121	484	70	M	E.faecium-B	PUO				S	R	R	R	S	S	S					
122	612	16	M	E.faecalis-WS	WOUND INFECTION	+			S	R	R	R	S	S	S					
123	790	61	F	E.faecalis-U	UTI	+			S	R	R	S	R	S	S					
124	844	18	M	E.faecalis-U	PUO	+			S	R	R	S	S	S	S					
125	712	56	F	E.faecium-P	ULCER				R	R	S	R	S	S	S					
126	784	14	F	E.faecalis-B	FFE	+	+	+	S	S	R	R	R	S	S					

127	935	23	M	E.faecium-B	PUO				S	S	R	R	R	S	S						
128	1216	48	M	E.faecalis-WS	CELLULITIS				S	R	S	S	S	S	S						
129	1129	67	M	E.faecalis-U	UTI				S	R	R	S	S	R	S						
130	1604	40	M	E.faecium-U	CKD				R	R	R	R	R	S	R	+	128	1	Van B	VanB
131	1638	48	F	E.faecalis-P	WOUND INFECTION				S	S	R	R	R	S	S						
132	1865	65	F	E.raffinosus-U	UTI				S	R	R	R	S	R	S						
133	1458	6	Mch	E.faecalis-B	PUO				S	R	R	S	R	S	S						
134	1971	35	M	E.faecalis-U	UTI	+	+	+	S	R	R	S	S	S	S						
135	1990	80	M	E.faecium-B	PUO				S	R	R	R	R	S	R	+	32	0.5	Van B	Van B
136	1989	50	M	E.faecalis-WS	CELLULITIS				S	S	R	R	R	S	S						
137	2164	27	M	E.faecium-WS	POST OP WI				R	R	S	R	R	S	S						
138	2415	49	F	E.faecalis-WS	WOUND INFECTION				S	R	R	S	S	S	S						
139	2119	19	M	E.faecalis-U	UTI				S	R	S	R	R	S	S						
140	2375	69	F	E.faecium-U	PUO				R	R	R	R	R	S	S						
141	2394	28	M	E.faecalis-U	UTI				S	R	R	R	R	S	S						
142	2452	69	M	E.faecium-U	PUO				R	R	R	R	R	S	S						

Ref. No. 68/E4/2/2014,

Govt. Rajaji Hospital,
Madurai.20. Dated:26.02.2014

Institutional Review Board / Independent Ethics Committee.
Capt. Dr.B. Santhakumar, M.D., (F.M.,) deanmdu@gmail.com
Dean, Madurai Medical College &
Govt Rajaji Hospital, Madurai 625020. **Convenor**

Sub: Establishment-Govt. Rajaji Hospital, Madurai-20-
Ethics committee-Meeting Minutes- for February 2014
Approved list - Regarding.

The Ethics Committee meeting of the Govt. Rajaji Hospital, Madurai was held on 07.02.2014, Friday at 10.00 am to 12.00.noon at the Anaesthesia Seminar Hall, Govt. Rajaji Hospital, Madurai. The following members of the committee have attended the meeting.

1.Dr.V. Nagarajan, M.D., D.M (Neuro) Ph: 0452-2629629 Cell.No 9843052029 nag9999@gmail.com	Professor of Neurology (Retired) D.No.72, Vakkil New Street, Simmakkal, Madurai -1	Chairman
2. Dr.Mohan Prasad , M.S M.Ch Cell.No.9843050822 (Oncology) drbkcmp@gmail.com	Professor & H.O.D of Surgical Oncology(Retired) D.No.32, West Avani Moola Street, Madurai -1	Member Secretary
3. Dr. Parameswari M.D (Pharmacology) Cell.No.9994026056 drparameswari@yahoo.com	Director of Pharmacology Madurai Medical College	Member
4. Dr.S. Vadivel Murugan, MD., (Gen.Medicine) Cell.No 9566543048 svadivelmurugan_2007@rediffmail.com	Professor& H.O.D of Medicine Madurai Medical College	Member
5. Dr.S. Meenakshi Sundaram, MS (Gen.Surgery) Cell.No 9842138031 drsundarms@gmail.com	Professor & H.O.D of Surgery Madurai Medical College	Member
6. Mrs. Mercy Immaculate Rubalatha, M.A., Med., Cell. No. 9367792650 lathadevadoss86@gmail.com	50/5, Corporation Officer's quarters, Gandhi Museum Road, Thamukam, Madurai-20	Member
7. Thiru..Pala. .Ramasamy , BA.,B.L., Cell.No 9842165127 palaramasamy2011@gmail.com	Advocate, D.No.72.Palam Station Road, Sellur, Madurai -2	Member
8. Thiru. P.K.M. Chelliah ,B.A Cell.No 9884349599 pkmandco@gmail.com	Businessman, 21 Jawahar Street, Gandhi Nagar, Madurai-20	Member

The following Projects was approved by the committee.

Name of P.G.	Course	Name of the Project	Remarks
Dr. R. Beaula Lilly beaulalrobert@gmail.com	PG in M.D., (Microbiology) Madurai Medical College, Madurai.	A study of the Biofilm formation and Vancomycin Resistance in a clinical isolates of Enterococci species in a Tertiary care centre	Approved

Please note that the investigator should adhere the following: She/He should get a detailed informed consent from the patients/participants and maintain it Confidentially.

1. She/He should carry out the work without detrimental to regular activities as well as without extra expenditure to the institution or to Government.

2. She/He should inform the institution Ethical Committee, in case of any change of study procedure, site and investigation or guide.

3. She/He should not deviate the area of the work for which applied for Ethical clearance.

She/He should inform the IEC immediately, in case of any adverse events or Serious adverse reactions.

4. She/He should abide to the rules and regulations of the institution.

5. She/He should complete the work within the specific period and if any

Extension of time is required He/She should apply for permission again and do the work.

6. She/He should submit the summary of the work to the Ethical Committee on Completion of the work.

7. She/He should not claim any funds from the institution while doing the work or on completion.

8. She/He should understand that the members of IEC have the right to monitor the work with prior intimation.

Member Secretary Chairman
Ethical Committee

26.2.14
DEAN/Convenor
Govt. Rajaji Hospital,
Madurai- 20.

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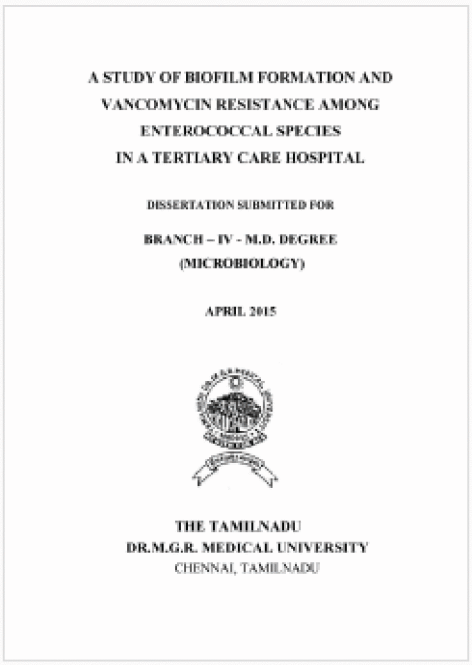

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ENTEROCOCCAL SPECIES
IN A TERTIARY CARE HOSPITAL

DISSERTATION SUBMITTED FOR

BRANCH – IV - M.D. DEGREE
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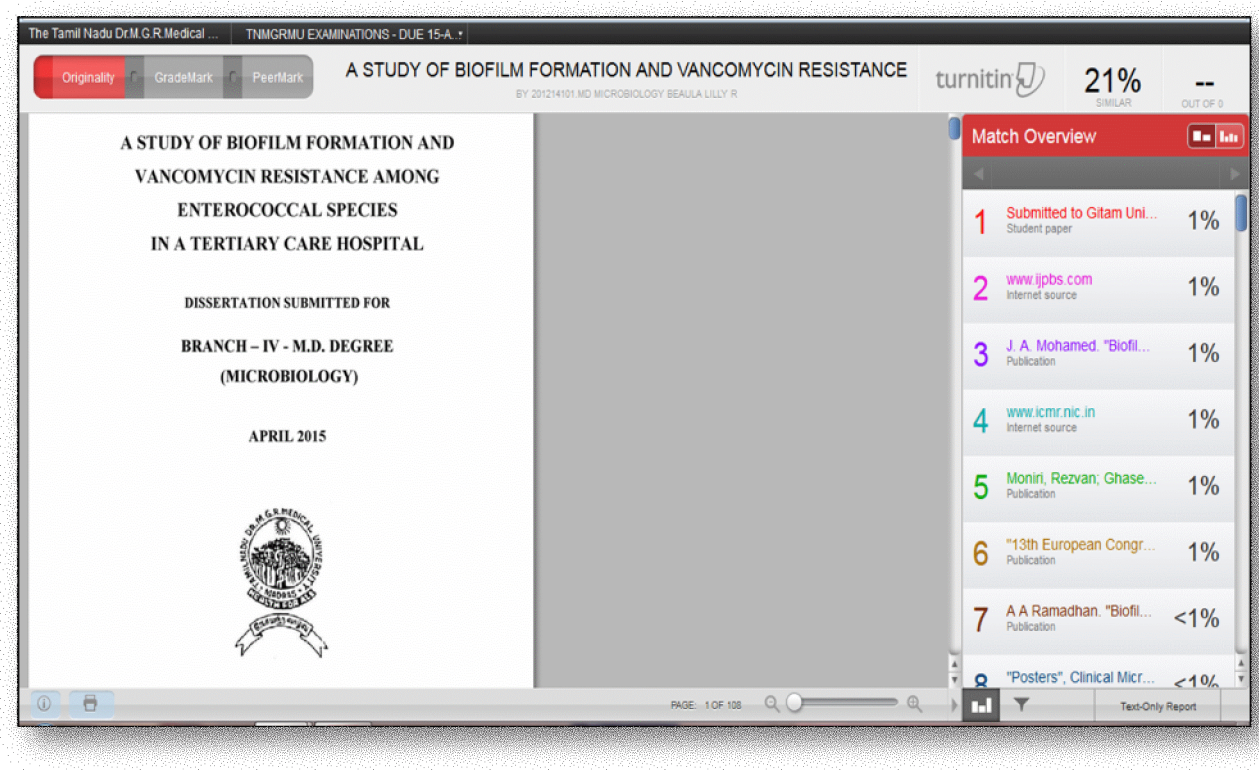
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